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On the Postnatal Development of Postural Mechanisms as Revealed by Electromyography and Myography in Decerebrate Kittens

By

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Abstract

SKOGLUND, S. *On the postnatal development of postural mechanisms as revealed by electromyography and myography in decerebrate kittens.* Acta physiol. scand. 1960. *49*. 299—317. — The present paper is one in a series of studies on the postnatal development of postural reflex activity in the kitten. Its aim is to investigate the reactions to decerebration and additional ablation of the anterior lobe of the cerebellum in these animals from birth until they are able to walk and stand. An attempt was made by electromyography and myography to trace the mechanisms behind the different reactions. It is shown that alpha rigidity is present in the newborn kitten while gamma rigidity develops later, appearing first in the forelimbs around a fortnight after birth and in the hindlimbs after three weeks. The missing gamma rigidity in the newborn animal is readily explained by the observation that no tonic stretch reflexes, as revealed by electromyography can be obtained from the muscles concerned with decerebrate rigidity. The tonic stretch reflex develops in a proximo-distal direction in the limbs as revealed by electromyography and is earlier obtained in the alpha than the gamma animal and earlier in the forelimbs than the hindlimbs. This development thus precedes the gamma rigidity which in its development closely follows that of the animal's ability to use its limbs for walking and standing. It also runs in a cranio-caudal direction.

From time to time the postnatal development of the nervous system has attracted considerable attention. Many attempts have been made to correlate function with various morphological signs of maturity, as for instance myeliniza-

tion (TILNEY and CASAMAJOR 1924, LANGWORTHY 1929). It is above all the postnatal development of the motor systems that has been used for these correlations. Most investigations were, however, performed in the first decades of this century (see LANGWORTHY 1929, WINDLE 1940, and BARRON 1941). Since then a wealth of mainly neurophysiological information has been gained in the adult animal, leading to a better understanding of the mechanisms involved in a motor act and postural reactions (see GRANIT 1955 and DOW and MORUZZI 1958). For the problems dealt with in this study the following facts, which make a reinvestigation of the postnatal development of postural reactions necessary, seem particularly relevant.

In his classical paper 1898 SHERRINGTON pointed out that the decerebrate rigidity described by him, which disappears on deafferentation and is thus driven by the stretch reflex, might not be the only kind of extensor hypertonus. LILJESTRAND and MAGNUS (1919) also found a decerebrate rigidity in completely deafferented forelimbs after transection of the spinal cord in the thoracic region and pointed out the importance of neck and labyrinthine impulses for creating extensor hypertonus. In 1931 POLLOCK and DAVIS showed that an extensor rigidity induced by tying both the carotids and basilar arteries persists after deafferentation. STELLA (1944 a, b) recalling that the procedure used by POLLOCK and DAVIS deprived the anterior lobe of the cerebellum of its blood supply, found that cooling or otherwise inactivating the anterior lobe in a decerebrate animal produced an extensor hypertonus in previously deafferented forelimbs. CARDIN (1946) later showed the same effect in the hindlimbs. It remained for ELDRED *et al.* (1953) and GRANIT *et al.* (1955) to show the real nature of these two types of rigidity. In the classical intercollicular preparation the decerebrate rigidity, as shown by these authors, is largely driven by the gamma loop operating the stretch reflex. In a decerebrate preparation where the anterior lobe of the cerebellum has been inactivated, the rigidity is induced by the alpha motoneurones alone without the support of the stretch reflex. GRANIT (1955) coined the expression alpha and gamma rigidity for these two types of extensor hypertonus. It has later been shown by MATTHEWS and RUSHWORTH (1957) that the tonic stretch reflexes in the intercollicular preparation disappear if the gamma fibres are blocked by procaine, confirming ELDRED *et al.* (1953). Alpha rigidity, though not caused by muscle spindle afferents, may simulate a reflex because the muscle contraction increases with extension (see GRANIT 1958, MATTHEWS 1959 a, b). This "pseudoreflex" has recently been studied by POMPEIANO (1960) in so-called alpha animals.

These new concepts must now be taken into consideration when evaluating previously recorded findings on the postnatal development of postural reactions as revealed by decerebration. Those findings will also be discussed in the light of the experimental results reported here.

A study of the development of postural reactions is for several reasons attractive. In the first place this development is readily observed in the ability

Fig. 1. A paramedian section of the brainstem and cerebellum in a 7-days old kitten. Note magnification 2×.

to walk and stand. Selecting an animal in which these abilities are gradually developed postnatally, offers the advantage of watching the development without any interference with normal conditions. By luck the commonly used experimental animal in neurophysiology, the cat, has such a postnatal development. To serve as a background for this and further studies a short description of its general development will be given under Results.

In the experiments presented here, some of which have been published in a preliminary form (SKOGLUND 1959 a, b) all postural reactions are studied as muscular performance. For a first analysis electromyography and myography have been used, and in that way all reflexes regardless of whether they employ the direct alpha or indirect gamma route to the muscle have been investigated. The question of the functional maturation of the different routes in postnatal development will thus be one of the main problems of this paper. Later work in course of preparation will deal with the postnatal development of afferent and efferent pathways (SKOGLUND 1960 a, b).

Material and Methods

Kittens from 30 different litters ranging in age from 1—45 days have been used. The results are based on successful experiments in 64 animals. The kittens were first studied in unrestrained conditions with respect to their ability of righting themselves, moving around, standing and walking. Thereupon they were subjected to various operations.

Most of the animals were decerebrated by section or suction between the superior and inferior colliculi under ether anesthesia given through a tracheal cannula. The carotid arteries were always tied. In some experiments the basilar artery was tied instead of performing a brainstem section (POLLOCK and DAVIS 1927).

In most animals subjected to intercollicular decerebration the anterior lobe of the cerebellum was removed by suction at a later stage of the experiment, while the animal was given additional ether. The level of transection of the brainstem is, as pointed out by WINDLE (1929), of importance for the production of decerebrate rigidity in the newborn kitten. In Fig. 1 the brainstem of a seven days old kitten is reproduced from a toluidin-stained section magnified twice. It is immediately obvious that on account of the size of the brainstem it is very difficult to produce exact localization by a transection. Furthermore, ventrally at the upper border of the pons the exit of the superior cerebellar arteries is situated. Interference with the blood supply to the anterior lobe of the cerebellum when the section is too far caudalward must be kept in mind. To avoid this,

most decerebrations have been performed by suction through a fine glass-pipette which leaves the vascular bed intact. In all experiments the level of transection of the brain-stem has been controlled on frozen or toluidin-stained material. On the other hand the extent of the cerebellar lesion has not been controlled, but judging from the results it has not been restricted to the cortex of the anterior lobe.

In many animals laminectomy was performed, either in the cervical or the lumbar region to allow sectioning of the dorsal roots or spinalization at Th 10.

In most animals various muscles in the hindlimbs, usually the gastrocnemius-soleus, tibialis anticus or quadriceps were dissected free. The hindlimb was fixed at hip and knee with drills in the usual manner. Sometimes the triceps muscle in the forelimb was dissected free in the same manner and the leg fixed at the elbow and the shoulder. The tendon of the muscles was connected to a strain gauge myograph for recording isometric tension. The electromyogram was recorded by steel needles (insulated to the tip) thrust into the muscle bellies.

The electromyogram was recorded either in isolated muscles together with the myogram or alone in undissected muscles. The reason for the latter procedure was twofold. In the first place it is difficult to dissect and connect *e. g.* the iliopsoas muscle to a myograph in a small kitten without destroying it. Secondly, dissection of the muscle might interfere with its blood supply and the muscle will be cooled. This is known to cause serious disturbances in the firing of the muscle spindles, which even can be silenced by cooling of the muscle. Rectal and muscle temperature were continuously controlled by thermocouples and the animal placed on a heated table and warmed by lamps. Dissected nerves and roots were kept covered by paraffin oil at 37° C.

Electromyogram and myogram were displayed by two double-beam cathode rays, one of which also gave time, and photographed on continuously running bromide paper.

Results

The postnatal development of the kitten's motor abilities:

The unrestrained newborn kitten does not walk or stand on its hindlegs and seems unable to support its body weight. It moves around by crawling movements with its forelegs, the abdomen trailing on the ground. It is born with closed eyes but has orientation in space. If put on its back it immediately tries to regain an upright position. When the animal is kept lying on its back one can observe that its limbs are flexed and adducted. When pinching the tail the animal suddenly rises on its hindlimbs and tries to escape. This shows that its inability to achieve an upright position cannot be explained by a failure of the efferent link from the ventral horn cells to muscle or of the muscle function.

After 7—9 days when the eyes open up the kitten has made considerable progress in its ability to move. From this time on its starts using its hindlegs in slow movements, still not being able to right itself and lift the abdomen from the ground. After the second week the first signs of an upright position and walking with the hindlegs become apparent. An invariable phenomenon accompanying these first walking and standing trials is an intentional tremor with slow frequency. This phenomenon is still observable at a later stage of development around three weeks postnatally when the kitten usually can

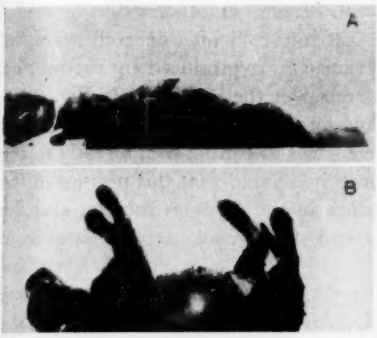

Fig. 2.

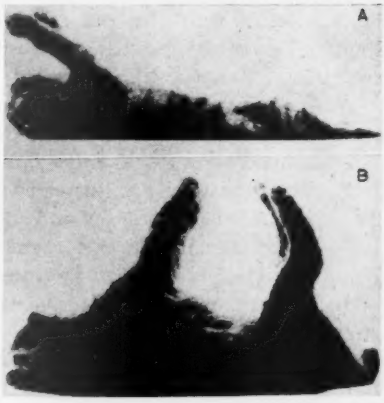

Fig. 3.

Fig. 2. Newborn kitten. In A after intercollicular decerebration by suction. In B after a subsequent ablation of the anterior lobe of the cerebellum (for further explanation see text).

Fig. 3. 12-days old kitten. In A after intercollicular decerebration. In B after a subsequent ablation of the anterior lobe. Note opisthotonus and the strong rigidity in the forelimbs.

stand and walk though with rather slow movements. Now, however, a more conspicuous trait is a sudden loss of tone in the hindlimb muscles every now and then which makes the gait uncertain and atactic. At the end of the fourth week the kitten walks rather well and is even able to run a few steps. From the fifth week on the kitten's ability to run is rather good. At the end of the sixth week only slight differences from a fully grown animal with regard to motor abilities are encountered.

There are of course great individual variations in this development even amongst kittens from the same litter. Thus there are animals with excellent walking abilities during the third week postnatally. Neither body weight, which has been routinely controlled, nor postnatal time is a good measure of the developmental stage of an animal.

After the kittens had been observed for some time in unrestrained conditions they were decerebrated and subjected to the various operations described under Methods. Although electromyography and myography were applied in most experiments, the general reactions of the animals in terms of extensor or flexor activity observed by inspection and resistance to passive movements of the limbs will, for descriptive reasons only, be presented first.

Intercollicular decerebration: On tying the carotids under ether anesthesia in a newborn animal a clearcut extensor rigidity very often appears in the forelimbs and is sometimes well visible in the hindlimbs too, though usually much weaker there. This reaction fades away in a few seconds to a minute. The ether anesthesia usually wears off in less than half an hour after the operation

(cf. LAUGHTON 1926), as can be judged from the animal's reaction to skin stimuli. The kittens stand the narcosis very well and seem to need high concentrations of ether in the respiratory air to become anesthetized.

When in the newborn kitten the classical intercollicular decerebration, as checked later in sagittal sections of the brainstem, is produced by suction, no extensor hypertonus ensues either in the forelegs or the hindlegs (see Fig. 2 A). Instead there is usually an increased flexor tone in both fore- and hindlimbs. The kitten is lying with flexed and adducted legs as can be seen already in the unrestrained animal. On closer examination it is found that this posture in the hindlimbs is achieved by an increased tonus in the iliopsoas muscles and the adductors of the thigh. On trying to extend the leg, an active resistance to stretch is encountered.

Skin stimuli, like pinching or touching the decerebrated newborn kitten, set up movements in all four legs (WEED 1917). After the stimulus has ceased the movements sometimes continue for some seconds up to a minute but ultimately the animal comes to rest in its earlier attitude of flexion and adduction and, if unstimulated, does not move. Pinching one hindlimb is followed by withdrawal of that limb (the flexor reflex) and evokes a series of movements in the opposite hindlimb of flexion and extension ending in a contralateral flexor reflex. That is, the opposite limb resumes its previous posture, flexed and adducted, although the stimulus continues. Thus no contralateral extensor reflex is obtained, only a bilateral flexor reflex.

Transection of the spinal cord at Th 10 in a newborn kitten subsequent to intercollicular decerebration induces very little change in the animal's attitude (cf. MALCOLM 1955). Sometimes a slight increase in the flexor tone of the hindlimbs can be noticed. Apparently no extensor rigidity is induced in the forelegs as in the adult animal with deafferented forelimbs (Schiff—Sherrington reaction). If the skin innervated from the spinal cord below the transection is denervated by flaying the extremities and the trunk and then sewn on again to preserve the temperature of the limbs, the flexor tone is somewhat reduced. A later bilateral deafferentation of the lumbar region from L 1 — S 1 removes it totally. The flexor tone must therefore be driven from the hindlimb receptors.

The flexor attitude which is less developed in the forelegs diminishes with increasing age. About 12—14 days postnatally, the first signs of an increased extensor tone can be observed in the forelimbs following intercollicular decerebration. The flexor tone in the hip muscles of the hindlimb is then considerably reduced. At 14—16 days postnatally a clearcut extensor rigidity can be observed in the forelegs. (Fig. 3 A). Around a week later, on the 21st to 25th day postnatally, a slight extensor rigidity appears in the hindlimbs too. This rigidity is, however, immediately abolished by section of the dorsal roots. During this development the reactions to skin stimuli in the decerebrate preparation are successively reduced.

When the intended intercollicular interruption of the brainstem was per-

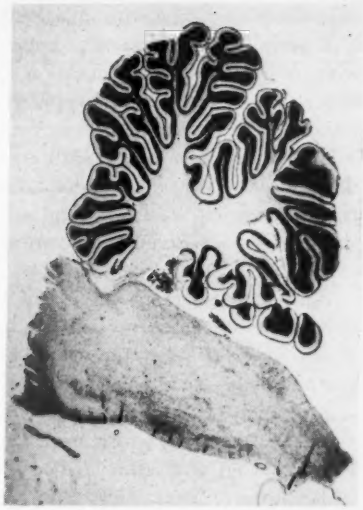

Fig. 4. A paramedian section of the brainstem and cerebellum in a 9-days old kitten showing the site of the brainstem section at the rostral border of the pons which gave extreme hypertonus in all four extremities. Magnification 4.5×.

formed by section and placed too far caudalward just in front of the pons ventrally as shown in Fig. 4 there often ensued an extensor hypertonus in the fore- and sometimes the hindlimbs in the newborn animal as described by WINDLE (1929). This preparation however, does not react otherwise with regard to deafferentation than the one to be described below.

Intercollicular decerebration with subsequent ablation of the anterior lobe of the cerebellum: When in the newborn kitten the intercollicular decerebration, as controlled later on sections of the brainstem, is followed by ablation of the anterior lobe of the cerebellum the flexor attitude in the limbs is replaced by a clearcut extensor hypertonus in the forelimbs and sometimes in the hindlimbs too. Invariably the flexor tone of the hindlimbs is reduced. Furthermore, in this preparation, as seen in Fig. 3 B, a clearcut opisthotonus is often observed. The animal lies with retracted head and has a strong resistance to bending in the neck muscles. This reaction points to an encroachment upon the roof nuclei of the cerebellum (BATINI, MORUZZI and POMPEIANO 1957). In testing the degree of extensor hypertonus in the newborn kitten by the resistance of the hind limbs to flexion, mere touching of the skin causes a reduction of the hypertonus and on bending the knee the resistance immediately yields (cf. WINDLE 1929). Touching or pinching the skin on the trunk or head on the other hand increased the extensor hypertonus, as found by POLLOCK and DAVIS (1931) in their anemically decerebrated and deafferented adult cat. The most effective way to influence the limb muscles, however, is to move the head up and down, while horizontal and rotating movements seem less effective.

In the preparation with ablation of the anterior lobe of the cerebellum there is in addition to the extensor hypertonus one striking difference from the one with intercollicular decerebration alone. Even if there is no clearcut extensor hypertonus in the hindlimbs, pinching one leg is promptly followed by a contralateral extensor reflex.

If in a newborn kitten, with good extensor rigidity induced by ablation of the anterior lobe or a caudal brainstem section, ipsilateral deafferentation is performed in either the cervical or the lumbar region, the decerebrate rigidity still persists unaltered and contralateral extensor reflexes are virtually unaffected.

With increasing age an extensor hypertonus in the hindlegs, induced by ablation of the anterior lobe in a previously intercollicularly decerebrated kitten, is invariably obtained. This begins to happen around 10—12 days postnatally.

An experiment repeatedly performed has been to spinalize previously decerebrated animals, which were too young to get extensor rigidity in the forelimbs after an intercollicular decerebration. As pointed out earlier, no reaction in the form of increased extensor tone in the forelegs is present. Thereafter a subsequent ablation of the anterior lobe of the cerebellum always gives a clearcut extensor hypertonus in the forelimbs. This is thus independent of the reflex to stretch. The time at which a decerebrated rigidity appears in previously deafferented forelimbs following spinalization has not been investigated. This reaction has only been seen once in a 44-days old kitten.

Anemic decerebration: Applying the anemic decerebration technique of POLLOCK and DAVIS (1927) gives the same results with regard to extensor hypertonus, its persistence after deafferentation and with regard to contralateral extensor reflexes, as does the intercollicular decerebration with ablation of the anterior lobe. In this preparation a certain degree of opisthotonus has always been observed (cf. POLLOCK and DAVIS 1930).

Electromyography and myography: For a first analysis of the events going on electromyography and myography have been used.

In an animal too young to show any extensor rigidity either in the fore- or the hindlimbs after an intercollicular decerebration it is always possible to record electromyographic and myographic activity in different muscles of the limbs in response to various stimuli. The electrodes must be rather small and a considerable amplification must be used. The muscles are very sensitive to cold and disturbances in their blood supply. Naturally the animals must be in excellent condition with regard to breathing and body temperature if any activity is to be obtained. To record the myographic activity a sensitive strain gauge is required. For recording muscular activity as small variations of tension the initial tension is important (see GRANIT 1958). After many trials it was found that variation of tension within certain limits does not add anything to the results obtained. Under too high initial tension (300—400 g) for some

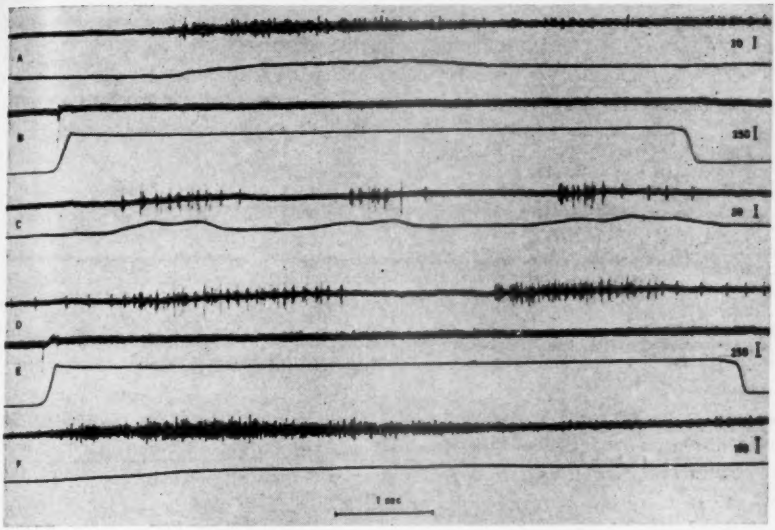

Fig. 5. Electromyograms and myograms in various muscles of 3-days old kitten. Myograph sensitivity in gr given in the picture. Light initial tension throughout. For further explanation see text!

time, however, the muscle deteriorated while tested by electrical shocks to the muscle nerve. Usually therefore rather light initial tension (50—100 g) has been used to have the muscles last through a whole experiment. The muscles are very plastic and the tension has to be readjusted some time after it has been set. The significance of this property of the muscle will be treated in connection with the activity of the stretch receptors (SKOGLUND 1960 b). When stretching the muscles, many different extensions were initially tried, but later, with increasing knowledge of the activation of the stretch receptors (SKOGLUND 1960 b), 5—10 mm was used.

Fig. 5 illustrates some responses obtained from various muscles in the hindlimb of a 3-days-old kitten, which had been decerebrated intercollicularly. In A is seen the response of the undivided gastrocnemius-soleus muscle on pinching the tail. In B the muscles are extended 8 mm but, in spite of increased amplification, no tonic stretch reflex is obtained. In C the response of the gastrocnemius-soleus muscles on pinching the opposite hindlimb is seen. A few bursts of activity appear, then the muscles are relaxed when the contralateral flexor reflex as described earlier sets in. In D the response of the undissected iliopsoas muscle on repeated stretching of the hip joint is recorded. The flexors of the knee, biceps-semitendinosus, did not give any stretch reflex, nor did the tibialis anticus. Then the anterior lobe of the cerebellum was removed and decerebrate rigidity appeared in both fore- and hindlimbs. Nevertheless no

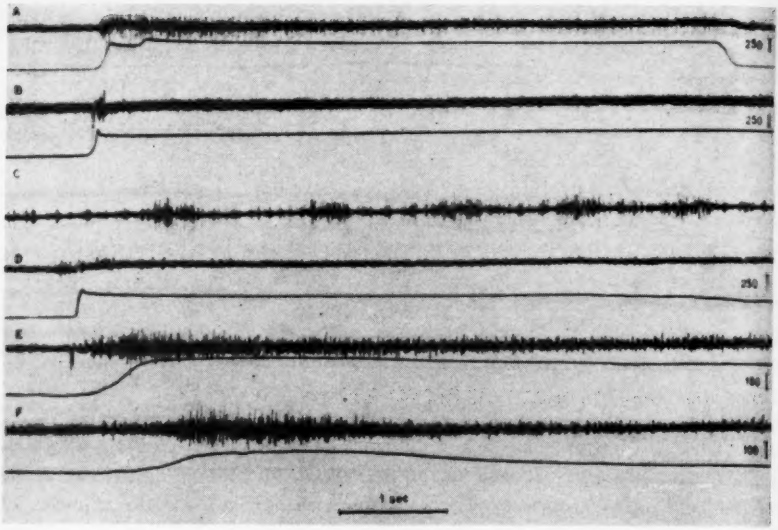

Fig. 6. Electromyograms and myograms from various muscles in 12-days old kitten. Myograph sensitivity in gr given in the picture. Amplification higher in B than in the other records. Light initial tension. For further explanation see text!

tonic stretch reflex could be recorded by electromyography in the quadriceps or, as seen in E, in the gastrocnemius-soleus muscles. On pinching the opposite hindlimb a contralateral extensor reflex appeared as shown in F in contrast to what happens in C.

The observations presented in Fig. 5 are typical for this age. It has never been possible to obtain tonic stretch reflexes from other muscles in the hind limb than the iliopsoas and sometimes from the adductors of the thigh, before the animals have reached the age at which decerebrate rigidity appears in the forelimbs after an intercollicular decerebration. In the forelimbs it is somewhat different. Repeatedly performed experiments on both dissected and undissected forelimb muscles in newborn animals have never given any tonic stretch reflex other than in the pectoralis muscle after intercollicular decerebration. After ablation of the anterior lobe, however, it has been possible to record a tonic stretch reflex in the triceps brachii already on the 6th day after birth. Being recorded by electromyography it must be considered a true reflex (cf. POMPEIANO, 1960).

Skin stimuli activate the flexor muscles especially in the hindlimbs where, as described earlier, the flexor tone in the newborn animal is rather pronounced. It has often been attempted to use the activation of the flexor motoneurones induced by skin stimuli as a background for setting up a tonic stretch reflex in these muscles but that has never been possible until about the stage of develop-

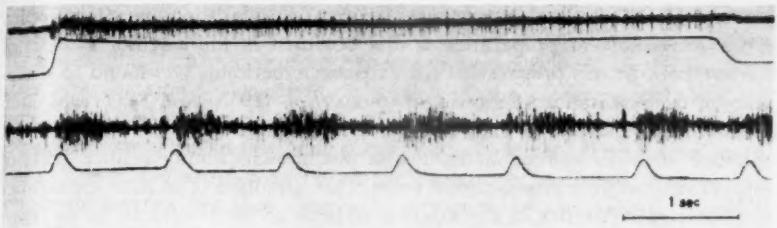

Fig. 7. 28-days old kitten intercollicularly decerebrated. The response of the gastrocnemius muscle to 8 mm stretch is seen in the upper record. In the lower the response to repeated tappings on the string joining muscle and myograph.

ment when a decerebrate rigidity appears in the forelimbs after intercollicular decerebration.

At the stage of development around 12—14 days postnatally, when the first signs of decerebrate rigidity appear in the forelimbs after an intercollicular section of the brainstem, a tonic stretch reflex is easily recorded in the triceps of the forelimb as seen in Fig. 6 A. Some days earlier when no decerebrate rigidity has appeared and some flexor tone can still be observed in the forelimbs it is often easier to record a stretch reflex in the biceps than in the triceps. From this, in combination with the earlier described activity in the pectoralis muscles, one is apt to conclude that the tonic stretch reflex is earlier developed in the flexors than in the extensors. That is, however, not the case as will be seen later from the experimental results in the hindlimbs where it is easier to demonstrate.

In Fig. 6 B is seen that the gastrocnemius-soleus muscles give no response to stretch in the intercollicular preparation. In C the response of the undissected iliopsoas muscle on repeated extension of the hip joint is shown for comparison. Then the anterior lobe of the cerebellum was removed and now a small tonic stretch reflex could be recorded in the gastrocnemius-soleus muscles as shown in D. In E is shown the strong crossed extensor reflex and in F the response of the gastrocnemius-soleus muscles on bending the head down.

In such experiments, as shown in Fig. 6, when the animals are around 10—12 days old it was often possible to record a tonic stretch reflex in the flexors but not in the extensors after intercollicular decerebration. After ablation of the anterior lobe the reverse was found, tonic stretch reflexes in the extensors but not the flexors.

At an even later stage of development, when the decerebrate rigidity is about to appear in the hindlimbs after intercollicular decerebration (21—25 days postnatally), as described earlier, a tonic stretch reflex can be recorded in the extensors of the hindlimbs, even before a clearcut extensor hypertonus can be observed. After 20 days postnatally the tonic stretch reflex is invariably obtained after intercollicular decerebration as seen in Fig. 7.

From the experiments illustrated in Fig. 6 is seen that the tonic stretch reflex in the intercollicular preparation is first obtained in the extensor muscles of the forelimb. In this preparation the extensor hypertonus was found to disappear on deafferentation. Following ELDRED *et al.* (1953) and MATTHEWS and RUSHWORTH (1957) it is then safe to conclude that the tonic stretch reflex obtained requires gamma activation of the muscle spindles, since without gamma support only phasic stretch reflexes are obtained. The later appearance of a tonic stretch reflex in the extensor muscles of the hindlimbs in an intercollicular preparation thus leads to the hypothesis that there is a cranio-caudal development of the gamma control. Keeping now to the hind limbs, the tonic stretch reflex is first obtained in the iliopsoas muscle of the hip as shown in Fig. 5, certainly a flexor. But at that stage of development (shown in the figure) no other muscle of the limb could be activated by stretch. Not even after ablation of the anterior lobe which gave a clearcut extensor rigidity in the hindlimbs could a tonic stretch reflex be obtained by electromyography. In the experiment illustrated in Fig. 6 on the other hand a small tonic stretch reflex could be recorded in the gastrocnemius after ablation of the anterior lobe as seen in D. From this can be concluded that there is a proximo-distal development of the tonic stretch reflex in the limbs. It might then be asked if this development is exclusively confined to a proximo-distal development of the gamma mechanism.

The tonic stretch reflex could be elicited somewhat before the time at which a decerebrate rigidity was clearly seen in the intercollicular preparation. The explanation for this at the stage of development when a decerebrate rigidity is about to occur is that electromyography is sensitive enough to catch the first traces of this reaction before all elements contributing to the massive response (of decerebrate rigidity) have developed. The tonic stretch reflex in the extensors of the hindlimb are, however, first obtained after ablation of the anterior lobe, as seen in Fig. 6. At the stage of development illustrated in this figure it was often possible, as described, to obtain a tonic stretch reflex in the flexor muscles in the intercollicular preparation. As shown by GRANIT *et al.* (1955) ablation of the anterior lobe leads to some paralysis of the gamma mechanism. The gamma support ought thus to be at its best for the extensor triceps muscle in the intercollicular preparation, and yet the tonic stretch reflex does not come off. The gamma control of a flexor muscle ought not to be high in the intercollicular preparation and yet the tonic stretch reflex is obtained. It is thus remarkable that the tonic stretch reflex is first obtained under the most unfavourable conditions for gamma excitation of the muscle spindles in respective muscles, suggesting that very strong alfa facilitation is present, strong enough to make the weak tonic spindle discharge maintain the reflex contraction (cf. MATTHEWS 1958 and 1959 b). Ablation of the anterior lobe in the cerebellum facilitates the tonic extensor motoneurones according to GRANIT *et al.* (1956) and in the intercollicular preparation the flexor activity was found to be

predominant before an extensor hypertonus appears. Thus the excitability of the motoneurones, flexor or extensor, will decide if the stretch reflex comes off. The actual appearance of an extensor hypertonus even in the newborn kitten after ablation of the anterior lobe shows that the efferent path from the ventral horn cells to muscle is functioning and the demonstration of a contralateral extensor reflex in this same preparation shows that the extensor motoneurones according to GRANIT *et al.* (1957) can be facilitated from various peripheral sources, yet they cannot be activated by the stretch receptors until at a certain time after birth as found by electromyography.

When later this activation appears, it turns up under the most unfavourable conditions for a gamma activation of the stretch receptors and long before a decerebrate rigidity depending on stretch reflexes under gamma control appears. This suggests the conclusion that there is a postnatal development of the afferent link from muscle spindle to ventral horn cell preceding development of the gamma mechanism. If this development is confined to the stretch receptor or its central connections or to both cannot be decided from these experiments.

In a later paper it will be shown that gamma control of individual muscle spindles can be demonstrated from about 17—20 days postnatally (SKOGLUND 1960b).

Discussion

The findings regarding the postnatal development of decerebrate rigidity in the intercollicular preparation are in good agreement with those of WEED (1917) and LANGWORTHY (1924). Both these authors found that the decerebrate rigidity appeared first in the forelimbs around 14 days postnatally and later in the hindlegs around 25—30 days after birth. The results reported here are on the other hand different from those of LAUGHTON (1926), who found extensor rigidity in the forelimbs of newborn kittens while the extensor hypertonus of the hindlimbs did not appear until after 6 weeks. The observations of WINDLE (1929) that an extensor hypertonus is present in both fore- and hindlimbs already in the newborn kitten, provided that the transection of the brainstem was performed at a certain level near the rostral border of the pons, are confirmed here in those cases when the decerebration was performed by section and by mistake placed too far caudalward. The preparation then obtained, however, did not differ from the one with ablation of the anterior lobe subsequent to an intercollicular transection of the brainstem performed by suction.

The level of transection which WINDLE found producing decerebrate rigidity in the newborn kitten will pass so near the exit of the superior cerebellar arteries that disturbances of the blood supply to the anterior lobe of the cerebellum can hardly be avoided. In investigating the postnatal occurrence of decerebrate rigidity in newborn rabbits GRIFFIN and WINDLE (1931) found that a transection of the brainstem including the rostral third of the pons

invariably induced extensor hypertonus and in this case the superior cerebellar arteries must have been injured. In this connection the observation made during this investigation of a transient decerebrate rigidity on tying the carotids in the newborn animal deserves attention. This phenomenon is probably due to the fact that the posterior cerebral and superior cerebellar arteries, which in the adult stage receive their blood from the basilar artery, seem to appear before this latter artery is formed during the development (PADGET 1948). The posterior communicating arteries remain relatively big for a long time and some of the blood to the cerebellum might still after birth be shunted that way. Closing the carotids would then induce a transient anemia of the anterior lobe of the cerebellum until the basilar artery takes over the nutrition.

The conclusion that must be drawn from the results obtained by ablation of the anterior lobe is that the cerebellum exerts a tonic inhibitory influence on vestibular mechanisms (see DOW and MORUZZI 1958), which as suggested by the righting movements observed here and also seen by WINDLE and FISH (1932), are in action already at birth. The vestibulospinal pathway is among the first to reach maturity (TILNEY and CASAMAJOR 1924). That the cerebellum exerts a tonic inhibitory influence already at birth has been denied by CHIARUGI and POMPEIANO (1954) and DE RENZI and POMPEIANO (1956). The findings of these authors will be considered here because what their observations — like those of LAUGHTON (1926) and WINDLE (1929) — in the light of recently obtained results by BATINI *et al.* (1957) really show is, as found here, that the cerebellum has a tonic inhibitory influence in the newborn kitten.

DE RENZI and POMPEIANO like CHIARUGI and POMPEIANO found a symmetrical extensor hypertonus in the forelimbs when they performed inter- or postcollicular decerebrations. The reactions to different types of decerebration are described in the paper of CHIARUGI and POMPEIANO (1954). Out of a total of 30 kittens from 1—45 days 18 were decerebrated intercollicularly, but the age of the kittens in this group is not given. The authors state that the animals showed a certain amount of extensor rigidity. A total of six kittens were decerebrated postcollicularly and showed strong decerebrate ridigity. In a total of 14 kittens from 1—17 days decerebrated either inter- or postcollicularly, CHIARUGI and POMPEIANO could not find any influence on the extensor tonus by stimulation of the anterior lobe. From 18 days on such a reaction was constantly found. DE RENZI and POMPEIANO (1956) prepared inter- and postcollicular kittens by the same method as CHIARUGI and POMPEIANO and could not produce any postural asymmetry by ablation of Larsells lobules III, IV and V in kittens between 1—18 days from birth but after that it was constantly found. A postural asymmetry of opposite laterality was constantly found in kittens from the 13th day when the unilateral lesion was deepened to encroach upon the fastigial nucleus, as shown by SPRAGUE and CHAMBERS (1953) in adult cats. From these experiments the authors concluded that the tonic inhibitory influence of the vermian part of the anterior lobe on postural

mechanisms begins 17—18 days after birth and that of the fastigial nuclei somewhat earlier on the 12th—13th day after birth.

The key to these experimental results appears to be the finding of an extensor hypertonus even in the newborn animal. The Italian workers cite LAUGHTON (1926) and WINDLE (1929) but do not consider the possibility that especially their postcollicular decerebration might disturb the blood supply of the cerebellum. In fact, CHIARUGI and POMPEIANO in their description of the decerebrate rigidity following postcollicular decerebration state that the cerebellar sign of opisthotonus was present. The same observation was also made by WINDLE (1929) when he got decerebrate rigidity following a low section of the brainstem. Also LAUGHTON points out that the muscles of the neck were involved in the decerebrate rigidity which he obtained. The reaction of opisthotonus does not appear unless the roof nuclei are destroyed (BATINI *et al.* 1957) or functionally inactivated by anemic decerebration (POLLOCK and DAVIS 1930). The superior cerebellar artery supplies the roof nuclei. Thus the rigidity found by these authors in the newborn animal must be of the alpha type (GRANIT 1955). Serious objections can thus be raised to the interpretations of CHIARUGI and POMPEIANO and DE RENZI and POMPEIANO in which the functional state of the cerebellum was uncontrolled and the type of decerebrate rigidity they obtained, alpha or gamma, was undetermined. It is, however, interesting to note that the postural asymmetry that could be induced from the fastigial nuclei and which is due to crossed inhibition (MORUZZI and POMPEIANO 1957) appeared at a time when tonic stretch reflexes, as found in this investigation, come into action.

All considerations regarding the level of transection of the brainstem for the production of a decerebrate rigidity are, of course, important and certainly deserve further experimentation as to the role played by different central structures. Furthermore the problem of variation in time from birth when decerebrate rigidity and walking abilities appear in different animals will have to be solved in terms of some objective measurement of neural maturation.

Keeping in mind that Sherrington in his classical description 1898 pointed out that "the decerebrate rigidity seems" to use his own words, "in some way dependent on the integrity of the afferent paths of the limbs", it is really astonishing how much interest has been focused on the postnatal development of the central structures involved in the production of decerebrate rigidity while the functional maturity of the stretch reflex has not attracted any attention as judged from the publications in the field. The experimental results presented here show, as far as they go, that whatever central structures are responsible for the extensor hypertonus that can be induced in the newborn kitten, it is brought about without the support of any tonic stretch reflex, thus being essentially an alpha type of rigidity (GRANIT 1955). Phasic stretch reflexes on the other hand might be present at birth, as indicated by MALCOLM's observation (1955) that rapid stretch gives a synchronous potential in the

ventral root (SKOGLUND 1960 a). It will remain for further studies to settle the questions about the postnatal development of the afferent link of the tonic stretch reflex (SKOGLUND 1960 b). Some of the observations made here, however, seem very important for such studies and will be commented on.

By ablation of the anterior lobe the balance between flexors and extensors was shifted in favour of the extensors. Subsequent transection of the spinal cord at Th 10 will naturally restore the predominance of the flexors. The dependence of the flexor-extensor balance upon supraspinal structures was shown in the adult animal by SHERRINGTON and SOWTON (1915) and has recently been studied by JOB (1953) and by ECCLES and LUNDBERG (1958). The intercollicularly decerebrated newborn kitten behaves with regard to flexor-extensor balance as if it was spinalized (also pointed out by MALCOLM 1955). This is further exemplified by the presence of a contralateral flexor reflex which as shown by RANSON and HINSEY (1931) can be obtained in the spinalized adult cat. Flexor reflexes are readily obtained in the hindlimbs of the kitten in the virtual absence of stretch reflexes other than in the iliopsoas muscle. From that one is apt to explain the increased flexor tone on the line that the flexor activity is not counteracted by any stretch receptor activity which will balance the flexors and extensors as in the adult animal. This explanation is also favoured by the gradual diminution of the flexor activity simultaneously with the gradual appearance of the stretch reflexes.

The development of the tonic stretch reflexes and the decerebrate rigidity after intercollicular decerebration first in the fore- then in the hindlimbs, points to a cranio-caudal development of the gamma mechanism. The proximo-distal appearance of the stretch reflex in the limbs depending on the development of the afferent link of the reflex could also mean a cranio-caudal development but confined to the cervical and lumbar regions respectively, proximal muscles having their nerve supply from the rostral parts of the intumescences. On the other hand it could also be a real proximo-distal development of the afferent link. With regard to the gamma fibres in the efferent link the following facts seem interesting. According to TORVIK and BRODAL (1957) the reticular formation should not send any direct fibres to the lumbosacral segments of the spinal cord which probably are activated via propriospinal neurones. These authors, however, showed that the reticulospinal fibres have reached L 1 in the 6-days old kitten. This suggests that at least part of the cranio-caudal development of the gamma mechanism might be confined to the intumescences, being a development of the propriospinal paths. On the other hand it could also be a proximo-distal development of the gamma fibres in the limb.

Summary

The postnatal development of some postural mechanisms in kittens from birth to 45 days of age has been studied. The ability of the animals to walk and stand has been correlated with their reactions to different types of decerebra-

tion and ablation of the anterior lobe of the cerebellum. The different types of decerebrate rigidity and reflex activities obtained at different ages have been analysed by deafferentation, electromyography and myography. Earlier findings are discussed at length.

1) At birth kittens are not able to walk or stand but have righting reflexes. They move around by crawling movements of their forelimbs. After a fortnight an upright position with the abdomen lifted from ground on walking can be observed, and a week later the kittens usually can walk. Still a week later, around 25—30 days, they take a few running steps. This development shows great individual variations.

2) Intercollicular decerebration does not induce any extensor hypertonus until around a fortnight after birth when it first appears in the forelimbs. Later, after three weeks, it appears in the hindlimbs. This rigidity disappears on deafferentation and is held to be a gamma rigidity. From this is concluded that the gamma mechanism develops in a cranio-caudal direction in the neuroaxis.

3) Ablation of the anterior lobe of the cerebellum subsequent to intercollicular decerebration induces a strong decerebrate rigidity with signs of opisthotonus regardless of the age of the kitten. This rigidity is most conspicuous in the forelimbs of a newborn kitten but often well visible in the hindlimbs also. Anemic decerebration consequently induces decerebrate rigidity in both fore- and hindquarters which, like the one induced by ablation of the anterior lobe, persists after deafferentation thus being an alpha rigidity.

4) It is concluded that the cerebellum exerts a tonic inhibitory influence on the vestibular nuclei already in the newborn kitten. Ablation of the anterior lobe of the cerebellum removes this inhibition and alters the flexor-extensor balance in the spinal cord from the previous flexor state in favour of the extensors. Thereby the reaction of the animal in response to a noxious stimulation of one limb is altered from a bilateral flexor reflex to a contralateral extensor reflex and ipsilateral flexor.

5) By electromyography it has been found that no tonic stretch reflexes can be elicited in the muscles concerned with decerebrate rigidity in the newborn animal. This explains the missing decerebate rigidity in the intercollicular preparation where it appears simultaneously with the tonic stretch reflexes.

6) By electromyography it is shown that the tonic stretch reflex is present in the proximal muscles of a limb at birth and appears later in the distal muscles. The tonic stretch reflex is obtained earlier in the alpha than the gamma animal as found by electromyography and must therefore be a true reflex, and earlier in the distal muscles of the forelimbs than those of the hindlimbs. From this is concluded that there is proximo-distal development of the stretch reflex in the limbs, confined to the afferent link of the reflex, preceding the development of the gamma mechanism, and being earlier in the forelimbs than the hindlimbs.

7) The postnatal development of the walking and standing abilities is related to the development of the decerebrate rigidity depending upon the stretch reflex.

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The Spinal Transmission of Proprioceptive Reflexes and the Postnatal Development of Conduction Velocity in Different Hindlimb Nerves in the Kitten

By

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Abstract

SKOGLUND, S. *The spinal transmission of proprioceptive reflexes and the postnatal development of conduction velocity in different hindlimb nerves in the kitten.* Acta physiol. scand. 1960. *49*. 318—329. — The postnatal development of the conduction velocity in different nerves of the hindlimb has been investigated in the kitten from birth to 4 months of age. It is found that the different fibre systems which have about the same conduction velocity at birth, with reference to conduction velocity, develop at different rates. Thus the large muscle afferents and efferents develop faster than the large fibres of the skin nerves and the latter faster than the gamma efferents. The conduction velocities reach adult values 3.5—4 months after birth. It is also shown that proprioceptive reflexes can be mediated by arcs of two neurons in the newborn kitten. Furthermore the reflex time for the two-neuron arc attains adult values when the conduction velocity of the fastest muscle afferents reaches 30 m/sec, which occurs around 20 days postnatally.

Experiments on the postnatal development of decerebrate rigidity in the kitten (SKOGLUND 1959 a, b, 1960 a) gave evidence that the tonic stretch reflex as revealed by myography and electromyography develops in a proximo-distal direction in the limbs. From the experimental findings it was further concluded that the appearance of this reflex is due to a development of its afferent link. The problem of whether a phasic stretch reflex is obtainable in these muscles was not investigated and the applied stretch was certainly not fast enough to set up such effects (cf. LLOYD 1943). MALCOLM (1955) however has succeeded in

recording a synchronous action potential from the central end of a cut ventral root in response to a brief stretch of hindlimb muscles. MALCOLM's results point to the existence of a phasic stretch reflex in the newborn kitten. Although MALCOLM used electrical stimulation of the muscle nerves in addition to stretch of the muscles, he did not investigate the time factors in the reflex arc, nor did he try to find out if the reflex really was monosynaptic or not. He stated that the response probably was monosynaptic, as it lacked an afterdischarge.

By using electrical stimulation of muscle nerves and roots, both peripheral conduction and central transmission of proprioceptive reflexes can be studied. By selecting nerves to distally situated limb muscles whose proprioceptive reflexes develop later postnatally than those of proximal muscles, it will be possible to follow the development over a considerably postnatal time and thereby probably to uncover what happens in the reflex arc during development.

It is of course of interest to see when the postnatal development with respect to conduction velocity is completed and some experiments have been made in kittens up to 4 months of age. The conduction velocities of a muscle and a skin nerve have also been compared at different stages of development.

Material and Methods

The experiments have been performed on kittens from 65 different litters ranging in age from 1—45 days. In addition some experiments have been performed on kittens up to 4 months of age. The animals were usually anaesthetized with Nembutal, 20—35 mg/kg body weight, given intraperitoneally. Some animals were decerebrated and spinalized.

Laminectomy was usually performed in the lumbar region of the cord and either all dorsal or all ventral roots from L II downwards were severed. This preparation was used for several types of experiments but here only those will be related in which the postnatal development of conduction velocity of the gastrocnemius and sural nerves were studied.

The nerves and roots were stimulated with square waves having a duration of 0.3—0.5 msec. The electrodes were silver silverchloride wires. The action potentials were recorded monophasically through a cathode follower input fed to a push-pull amplifier, displayed on a cathode-ray beam and photographed. Time was given by a sine wave generator.

The conduction velocity in the peripheral nerves was determined in the following way. The dorsal or ventral root was stimulated using shock strengths well above threshold for the first potential wave recorded from the peripheral nerve. The conduction time was measured from the beginning of the shock artefact to the foot of the first potential wave. The latter is often indistinct and to avoid the error introduced thereby the conduction time from the beginning of the shock artefact to the peak of the first potential wave — when a good many fibres certainly have fired — was also measured. The conduction velocity was then calculated using the conduction distance measured on the nerve from the cathode of the stimulating electrode to the recording one. In using both these conduction times when calculating the conduction velocities, two values are obtained between which the conduction velocity of the fastest fibres in the first potential wave is lying.

The time for spinal transmission was calculated in the following way. A muscle nerve or a dorsal root was stimulated and the reflex potential recorded on the ventral root or a muscle nerve. The afferent and efferent conduction times to and from the cord were then subtracted. The intraspinal conduction path was measured on frozen and on osmium stained sections of the spinal cord.

The animals were kept warm with lamps on a heated table. Rectal temperature was continuously controlled. All dissected nerves and roots were kept covered by paraffin at 37° C.

The methods used for determining the central reflex time and the conduction velocity are of course liable to errors. The slow conduction rate in the newborn kitten (HURSH 1939 a) being linearly related to the fibre diameter with a factor of 6 (HURSH 1939 b), makes errors of a few millimeters in the measured conduction distance and of tenths of milliseconds in the conduction time obtained, insignificant. When the conduction velocity between two nerves or types of fibres can be compared for a constant conduction distance, as is easily done in the experimental situation at hand — changing recording from the gastrocnemius to the sural nerve in the popliteal fossa or shifting stimulation from the dorsal to the ventral root, interelectrode distance being kept constant and the nerves slightly stretched — the method is an extremely sensitive one. Control measurements of fibre diameters have been made but that material, including also other nerves and roots than those used here, will be presented in another publication (SKOGLUND and VALLBO 1960). The reason for not trying to correlate the development of conduction velocity to the fibre spectrum is immediately obvious from the results. The morphological method is too insensitive compared with the physiological one and absolutely useless for ascertaining such small differences as are obtained when comparing muscle and skin nerves in the newborn animal. The linear relation between the fibre diameter and the conduction velocity with a factor of 6 means that variations within one micron would have to be compared. Furthermore a comparison between the fibre spectrum of the afferent in a muscle nerve and those of a skin nerve is obscured by the presence of the efferents in the former. A correlation between conduction velocity and the fibre size of different types of fibres in a muscle nerve is for obvious reasons not possible unless either afferents or efferents have been caused to degenerate by root section. During the period of 2—3 weeks necessary for degeneration the remaining fibres will continue to grow and thus at least the first period of postnatal development cannot be studied with this method. In addition the development of the remaining fibres might be altered in an unknown way.

On the other hand the reason for not including comparisons between the conduction velocity of nerves to proximal and distal muscles has been the errors of the physiological method. In nerves to proximal muscles conduction distance is very short and this tends to increase the error introduced by measurements of conduction distance and does so even more if the conduction velocity there were higher than in nerves to distal muscles. In the latter case errors from measuring conduction time would play a great role. As with proximal muscles the advantage of comparing identical conduction distances falls out, the morphological method with them is considered more reliable than the physiological, the more so the greater the difference between the nerves.

Results

Conduction velocity in peripheral nerves. At birth the conduction velocity of the fastest afferents in the gastrocnemius nerve varies from 9—12 m/sec probably depending upon how mature the animal is at birth. This seems to be different even amongst kittens of the same litter (SKOGLUND 1960 a). The conduction

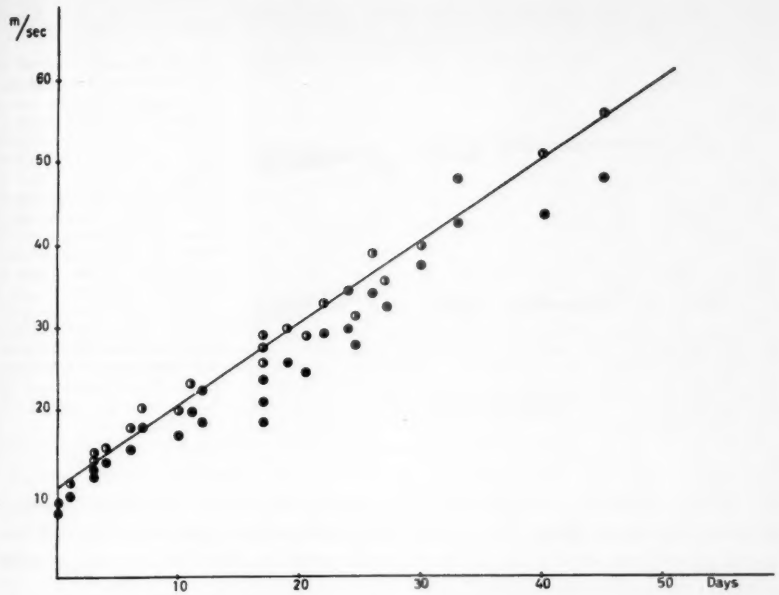

Fig. 1. The conduction velocity of the fastest afferents in the gastrocnemius nerve plotted against time in days postnatally, half filled circles. Filled circles, conduction velocity of the peak of the first potential wave. The regression line for the fastest fibres, y on x, fitted by the method of least squares: $y = 11.04 + 0.99\ x$. Correlation coefficient $r = 0.98$.

distance from the gastrocnemius muscle up to the spinal cord has been measured to 3.5—4.5 cm in newborn kittens. This means a conduction time of 3—5 msec for the fastest afferents from the gastrocnemius muscle.

With increasing age the conduction velocity increases by approximately 1 m/sec, daily reaching 30 m/sec around 20 days postnatally (see Fig. 1). At the same time the conduction distance from the gastrocnemius muscle to the spinal cord increases to 7—8 cm. The conduction time for the fastest muscle afferents for this path is now reduced to less than 3 msec.

The conduction velocity of the fibres contributing to the peak of the first potential wave (see Fig. 1) is immediately after birth slightly less than that of the fastest fibres, decreasing with increasing age. This must to some extent be due to the spreading out of the fibre spectrum on a scale of microns and is a rough estimation of the maturity of most of the fastest fibres.

The ventral roots are earlier developed than the dorsal roots and are earlier myelinated (see REXED 1944). In the newborn kitten the conduction velocity of the fastest muscle efferents has also been found to be 2—6 m/sec higher than that of the afferents. This difference diminishes with increasing age and

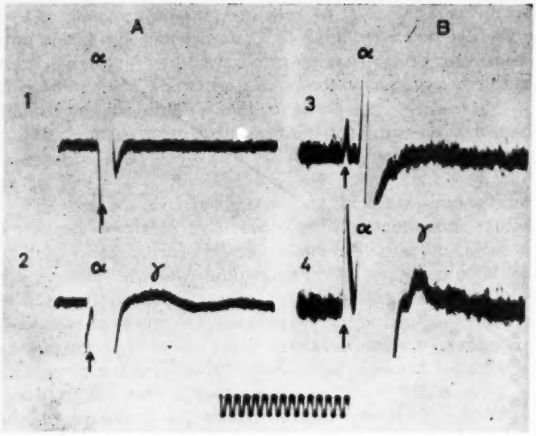

Fig. 2. Records to show a late wave in the neurogram of the gastrocnemius nerve on stimulation of the ventral root S I. A from 8-day-old kitten. B from 17-day-old kitten. In records 1 and 3 the ventral root is stimulated with a shock strength maximum for the first potential wave (alpha) in the neurogram. In records 2 and 4 stimulus strength is increased to 10 times threshold for the fastest fibres of the first potential wave obtained and the gamma wave appears. Superposition of 50 faint traces. Arrows indicate stimulus artefact.

the fastest afferents and efferents have about the same conduction velocity between 20 and 30 days. This is also seen in the calibre spectra of the roots but the biggest fibres in the dorsal root are fewer in number than in the ventral (SKOGLUND and VALLBO 1960).

The sural nerve has been found to conduct at the same velocity in the newborn animal as the gastrocnemius nerve when the conduction velocity is measured at the same distance from the cord. At a later stage of development, around 30 days postnatally, the conduction velocity is found to be about 10 m/sec slower in the skin nerve than in the muscle nerve. As a rule the conduction velocity after that time is always found to be lower in the sural than in the gastrocnemius nerve. Thus — to mention some experimental values — around 40 days the skin nerve conducts at 35 m/sec, as against 50 m/sec for the muscle nerve; and at two months the former conducts at 48 m/sec, and the latter at 71 m/sec. In a total of 9 animals ranging in age from 2 to 4 months the conduction velocity has been found to increase from 50 to 82 m/sec in the sural nerve and from 70 to 115 m/sec in the gastrocnemius nerve. The development of the conduction velocities is thus finished around 3.5—4 months after birth.

When stimulating the ventral roots at a strength of 10—15 times threshold for the fastest efferents, LEKSELL (1945) succeeded in obtaining his gamma efferent potential in the neurogram. In repeating LEKSELL's experiment with superposition of about 50 action potentials it has been possible, as seen in Fig. 2 (B 2), to obtain a delayed wave in the motorneurogram, conducted at around 8—10 m/sec, after ten days of age. In order to see this wave the stimulus strength had to be increased by as little as 5 times the threshold of the fastest fibres. The reason for the low threshold compared with the alpha wave will be

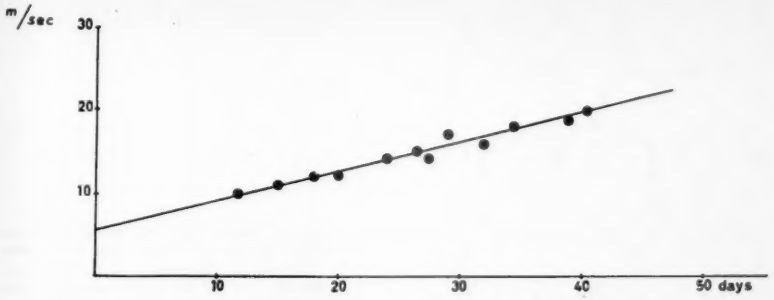

Fig. 3. Conduction velocity of the fastest fibres in the late wave (gamma) seen in Fig. 2 plotted against time in days postnatally in twelve experiments. Regression line y on x fitted by the method of least squares: $y = 5.61 + 0.35\ x$. Correlation coefficient $r = 0.98$.

taken up in the Discussion. Before ten days it is difficult to obtain any distinct delayed potential wave that allows any measurements of conduction time (see Fig. 2 A).

From the plot of the conduction velocity of the fastest fibres of the early wave against age (Fig. 1) it is seen that their conduction velocity from about 15 days up to 40 days postnatally increases from 25 to 50 m/sec, and that of the fastest fibres in the late wave (Fig. 3) from 10 to 20 m/sec. The rate of growth is given in the legends of Figs. 1 and 3. If the fibres continue to grow at these rates to full maturity which with regard to the afferents has been demonstrated to be true (see above) the conduction velocity of the fastest fibres will have to increase 2.4 times to reach 120 m/sec. The conduction velocity of the efferents in the later wave, would in this case be 49 m/sec. KUFFLER, HUNT and QUILLIAM (1951) have shown that the conduction velocity for the gamma efferents is 15—50 m/sec with a peak at 30 m/sec. It is thus highly probable that the later wave obtained in the neurogram of the efferents in the kitten corresponds to the gamma fibres of the adult stage. This conclusion is fully substantiated by the observation that, at a stimulus strength setting up the late wave the pause in the firing of a gastrocnemius spindle during extrafusal contraction can be filled in after 17—20 days postnatally (SKOGLUND 1960 b).

The spinal transmission of proprioceptive reflexes. On stimulating a muscle nerve or a dorsal root and recording from the ventral root, a fairly well synchronized potential is obtained (MALCOLM 1955) in the newborn kitten. With increasing age the reflex time diminishes, as seen in Fig. 4 where the records of the ventral root potentials from three different experiments are shown. For comparison of the conduction times the shock artefacts in the records have been placed on the same vertical line. In the left row the muscle nerves are stimulated at the distance from the spinal cord given in the legend and in the right row the dorsal roots are stimulated around 1 cm from the cord. As seen in the lowest records in

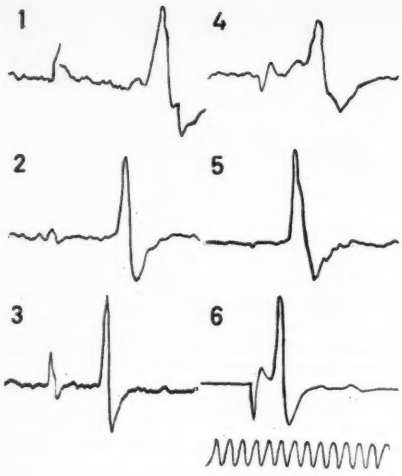

Fig. 4. Reflex potential recorded on central end of cut ventral root S I in three different experiments. 1, 2, and 3 on stimulation of the gastrocnemius nerve in the popliteal fossa, afferent nerve path 3.4 cm, 5.4 cm and 6.8 cm respectively. 4, 5 and 6 on stimulation of the dorsal root S I. Record 1 and 4 from 1-day-old kitten, 2 and 5 from 12-day-old kitten, 3 and 6 from 28-day-old kitten. Time 1,000 c/sec.

the 28-days animal the reflex time is reduced to 3.5 msec on stimulating the muscle nerve and to 1.5 msec on stimulating the dorsal root. The reflex times are then about the same as those obtained in the adult animal (RENSHAW 1940, LLOYD 1943).

The long reflex time as shown in Fig. 4 in the upper row of records (1 and 4) does not immediately appear to be mediated by arcs of two neurons. The flexor behaviour of the newborn animal (SKOGLUND 1960 a) might suggest that the reflex is polysynaptic and directed to the flexors. This possibility is immediately ruled out on stimulating and recording from the muscle nerve or stimulating the dorsal root and recording from the muscle nerve when a potential with the same central reflex time is obtained in extensors as well as flexors.

In Fig. 5 the reflex time on stimulating the dorsal and recording from the ventral root has been plotted against conduction velocity in the fastest muscle afferents in eleven experiments (circles) from different postnatal stages. Subtracting the conducting time in the dorsal and ventral roots in the same experiments by measuring the distance from recording and stimulating electrodes to the cord and calculating the conduction time from the velocity of the fastest afferents gives the curve with half filled circles. Measurements on spinal cord sections in newborn to 10-day old kittens have shown that the intraspinal conduction path is about 2 mm in the first sacral segment. Plotting the conduction time in the intraspinal path, assumed to be at length 2 mm in all experiments, against conduction velocity in the muscle afferents, intraspinal conduction rate being assumed to be a third of that value, gives the dots in Fig. 5. Two assumptions are here made. First that the intraspinal conduction velocity

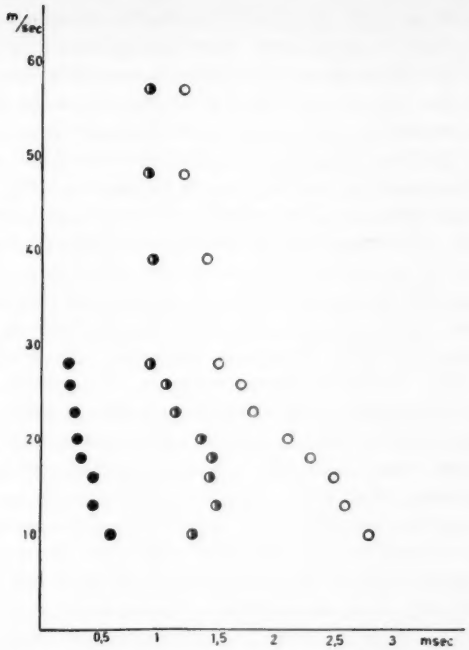

Fig. 5. *Reflex time* on stimulation of the dorsal root and recording on the ventral root S I plotted against the conduction velocity of the muscle afferents in eleven experiments (circles) at different postnatal stages. *The central reflex time* plotted against conduction velocity of the muscle afferents in the same eleven experiments (half filled circles). Intraspinal conduction time in the afferents plotted against conduction velocity of the muscle afferents up to 28 m/sec (dots). For further explanation see text!

of the afferents is a third of the maximal velocity of their extraspinal path. This seems justifiable because as low values as 12—20 m/sec are given by BROOKS and ECCLES (1947) in the adult cat, which is only one sixth of the maximum velocity for the fastest afferents. The second assumption which is that the conduction velocity of the dorsal root collaterals increases proportionately with that of the peripheral nerve, also seems justifiable since root collaterals are a direct continuation of the neurons.

From the data given in Fig. 5 (half filled circles), it is seen that with a synaptic delay of 0.5—0.8 msec there would be no time for more than one synapse because an additional one, as shown for instance by ECCLES, FATT and LANDGREN (1956), takes another 0.8 msec. Together with the extreme value for synaptic delay 1.0 msec (LORENTE DE NÓ 1938, RENSHAW 1940) this would require a delay of 1.8 msec which is nearly double the value obtained by subtracting the values of the dots from those given by the half filled circles

which will give the synaptic delay. The conduction velocity in the thin dorsal root collaterals might be even slower than the calculated value and the conduction path in the older animals somewhat longer than 2 mm which would shift the dots to the right. From these calculations it can be concluded that a reflex can be mediated by arcs of two neurons in the newborn animal. The long reflex time which is obtained on stimulating a dorsal and recording from a ventral root is thus in all probability mainly explained by the slow conduction velocity. A longer synaptic delay which with certainty can be determined only by the use of intracellular microelectrodes should, however, be considered.

It could be argued that on stimulating the dorsal root the potential obtained is set up by the contribution of afferents from proximal muscles, and that, consequently, those from the distal muscles might have a very much longer central reflex time. As stated above, however, the central reflex time was measured when recordning from the muscle nerve and was the same both when stimulating muscle nerve or dorsal root. It should be added that the central reflex time when stimulating the dorsal and recording on the ventral root S I is not shorter than when recording distally on the muscle nerve.

In Fig. 4 is seen that the reflex time in the 28-days animal is about the same as in the adult animal. In Fig. 5 is shown that the shortening of the reflex time after the fastest muscle afferents have reached 30 m/sec (around 20 days postnatally, see Fig. 1) is insignificant. Furthermore the intraspinal conduction time minus synaptic delay at that time is 0.25 msec (Fig. 4) which might be too low a value. The intraspinal conduction time in the adult animal as given by RENSHAW (1946) and BROOKS and ECCLES (1947) amounts to 0.25—0.35 msec.

Discussion

The results presented here on the postnatal development of the conduction velocity are with regard to the skin nerve in agreement with those of HURSH (1939 a). From his figures it can be seen that the conduction velocity of the saphenous nerve increases more slowly with time than that of the gastrocnemius nerve just as found here. Such a difference between muscle and skin nerves is also found when comparing the gastrocnemius and sural nerves. The conduction velocity of the muscle nerve increases about 0.99 m/sec daily and that of the skin nerve about 0.65 m/sec daily. These figures, obtained from the regression-lines fitted by the method of least squares are of course no exact values but, by showing approximately the rate of development, they are useful for comparisons. Calculating in the same manner, the conduction velocity of the gamma efferents will make them increase about 0.35 m/sec daily. The observation that functionally different fibre systems develop at different rates postnatally seems interesting and raises the question as to whether this is a general

principle. From the point of view of function it must be of the utmost importance, because, generally expressed, fibres cannot start functioning until at a certain degree of maturation. If all fibres developed at the same speed postnatally, those having in the adult stage a lower conduction rate, like the skin nerves, must then start to develop later to reach their lower final conduction velocity. Thus they could not start functioning until late after birth and that obviously is not true. Reflexes from the skin are readily obtained in the newborn animal (see SKOGLUND 1960 a). Another principle would be that the fibres stopped growing at different times postnatally. This supposition, like the former, is negated by the experimental observations. Before birth the development of the fibres must obviously be governed by factors which allow the skin nerves to reach about the same maturity as the muscle nerves at birth. This probably necessary for the functionally important nociceptive reflexes.

Fig. 2 showed that the delayed wave of gamma potentials differed much less in threshold from alpha fibres than is the case in adult animals. This is simply explained by the much smaller difference in conduction velocity between alpha and gamma fibres at about ten days postnatally, suggesting that the fibres have not yet undergone the necessary differentiation in size (cf. Fig. 2 and 3).

Extrapolating the experimental values for conduction velocity of the fastest gamma efferents gives 5—6 m/sec at birth. According to HURSH (1939 b) the conduction velocity of developing fibres is linearly related to their fibre diameter with a factor of six. When dividing the conduction velocity of the fastest fibres in the three fibre groups investigated, viz. muscle efferents, afferents and gamma efferents, it is found that the difference in fibre diameter is very small at birth. Calculating with the extreme values, 19 m/sec for muscle efferents and 6 m/sec for gamma efferents the difference is only 2 microns between these fibre types. To ascertain even smaller differences, such as those to be expected between the afferents of the gastrocnemius and sural nerves it is impossible to rely on calibre measurements in osmium stained material.

The results presented, that the reflex time diminishes with increasing age mainly due to the reduction of the peripheral conduction time, reaching about the values obtained in an adult animal when the conduction velocity of the fastest muscle afferents attains 30 m/sec, is completely contrary to the conclusion drawn by HURSH (1939 a).

HURSH correlated the development of the conduction velocity to the increasing length of the leg from the tip of the toe to the head of the femur and found that there is a linear relationship between the two. From that he drew the conclusion that the conduction time is constant in that slow conduction is 'nicely compensated' for by decreased length of the nerve path. However, his datas (his Fig. 2) show that at length 8 cm the conduction velocity is 10 m/sec at 18 cm as much as 60 m/sec. The conduction time in the first case is ob-

viously 8 msec as against 3 msec in the latter case. The additional conduction path from the head of the femur to the spinal cord will accentuate this difference unless it is 6 times longer in the older animal which cannot be true. This means that although the relationship between conduction velocity and fibre length is linear, the compensation of one by the other is by no means complete. It could be so only if the angular coefficient were unity which clearly is not the case. In reality the leg increases from 8 to 18 cm or 2.25 times as against an increase of 6 times for the conduction velocity (from 10 to 60 m/sec).

No systematic measurements of the conduction path from the gastrocnemius muscles to the spinal cord have been made. From the data at hand, together with the figures given by HURSH (1939 a), however, it is possible to calculate that about 80 % of the decrease in conduction time has occurred when the fastest fibres conduct at a rate of 30 m/sec. After that the decrease in conduction time is very slow up to the adult stage. It is interesting to note that the reflex time attains the adult value around 20 days postnatally, the conduction velocity being about 30 m/sec (see Fig. 1). This is at the time when a gamma driven decerebrate rigidity appears in the intercollicularly decerebrated kitten (SKOGLUND 1960 a). In a later paper it will be shown that this is the time when the muscle spindle by gamma stimulation can be made to fill out the so-called pause coinciding with the rising phase of the extrafusal contraction. This further substantiates the explanation proposed by GRANIT (1955) for the fast monosynaptic pathway: 'Half a millisecond one way or another would hardly as such be of any significance whatever for large limb muscles and bones of considerable inertia and momentum. But for the cerebral command to the loop it is essential that it be carried straight to the ventral horn cells by a *fast path* (italics here) not subject to complex polysynaptic influences.'

Another point that might be worth noticing is that the gamma efferents, at the time when to judge by true decerebrate rigidity (SKOGLUND 1960 a) they start functioning they conduct at 15—20 m/sec which is about the same as the muscle afferents do 5—10 days after birth.

From the experimental results presented here it is obvious that MALCOLM's finding of a monosynaptic pathway in the newborn kitten is in essential agreement with the present results. The explanation of the missing tonic stretch reflex in kittens below 17—20 days postnatally is thus not the absence of monosynaptic connections. It will be shown in work shortly to be published to what properties of the stretch reflex such findings refer. The preparation deals with muscle spindles and their innervation in postnatal development.

This work is part of a series of investigations into the postnatal development of the postnatal reflexes supported by the Swedish Medical Research Council.

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From the Pharmacological Institute, University of Uppsala, Sweden

Zinc-Induced Relaxation of Muscle Fibres

By

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Abstract

Edman, K. A. P. *Zinc-induced relaxation of muscle.* Acta physiol. scand. 1960. *49.* 330—342. — The extractability of zinc from rabbit psoas muscle by glycerol-extraction and by further treatment with EDTA and ATP has been determined as has the uptake of zinc by glycerol-extracted fibres at bath concentrations of zinc corresponding to the free zinc ion concentration needed for induction of complete isometric relaxation of the fibres in the presence of ATP. The functionally relevant uptake of zinc needed for relaxation of extracted fibres could be covered by the total amount of zinc available in fresh muscle. Fully repeatable contraction-relaxation cycles with glycerol-extracted fibres in a highly plasticized state were produced with 0.6 mM total zinc simply by alternating the ATP concentration between 3 mM (relaxation) and 7 mM (contraction). The result was similar if, at constant total zinc (0.6 mM) and ATP (3 mM), 1.25 mM EDTA was alternately added (contraction) and removed (relaxation). Based upon these findings a possible mechanism for the action of zinc as a relaxing factor in vivo is presented: Relaxation is caused by inhibition of contractility by zinc; contraction is induced by release into the appropriate compartment of the fibre of a zinc-complexing substance — ATP or a similar compound — which, after breakdown, again returns the complexed zinc with consequent re-inhibition of contractility and, thus, relaxation.

The relaxing effect of zinc in the presence of ATP[1] on glycerol-extracted muscle fibres has been reported in several previous papers (Edman 1956, 1958, 1959 a, 1959 b, 1959 c, 1959 d, 1960). In view of the comparatively high concentration of zinc in fresh skeletal muscle it is reasonable to assume that the

[1] The following abbreviations will be used: ADP, adenosinediphosphate; ATP, adenosinetriphosphate; EDTA ethylenediamine tetraacetate.

zinc effects obtained on the isolated contractile system might be of relevance for the contraction-relaxation process in the living cell. The present work includes experiments with glycerol-extracted fibres illustrating a possible mechanism of zinc-induced relaxation in living muscle. It will also be shown whether the zinc concentrations needed in the extracted muscle fibres for obtaining relaxation could be supplied by the zinc available in the living muscle cell.

Methods

Rabbit psoas muscle was used. For the glycerol-extraction of the muscle tissue see Methods I and II. The "veronal buffer solution" (EDMAN 1957) with pH 7.3 containing 100 mM potassium and 10 mM diethylbarbituric acid was used throughout as medium after the glycerol-extraction. In the contraction-relaxation experiments 1 mM magnesium was also included in the buffer solution. The medium used in the zinc analysis experiments did not contain magnesium if not otherwise stated.

$ZnCl_2$, pro analysi, E. Merck, diethylbarbituric acid (Veronal) according to Ph. S. IX (for analysis, see EDMAN 1958) and adenosintriphosphate, disodium salt, Pabst lot no. 116 A (for analysis, see EDMAN 1960) were used. All other chemicals used were of analytical grade. The water was redistilled in a borosilicate glass distiller. If not otherwise stated, the concentrations given in this paper refer to total concentration in the bath. For the calculation of free zinc in the bath the stability constants given earlier (EDMAN 1959 a) were used, viz. 4.00 for the MgATP complex and 4.76 for the ZnATP complex. All experiments were performed at room temperature, 20—22° C.

I. *Contraction-relaxation experiments*

The glycerol-extraction of the fibre bundles was described earlier (EDMAN 1957) as was the technique used for recording the isometric tension. The cross section of the fibre bundles was 89—117 × 168—210 μ and the length 10.0 mm. The deviation from ideal isometry was < 0.2 per cent of the fibre length. An initial tension of 15 mg was given to the fibre bundles. The veronal buffer solution with 1 mM magnesium was used as medium, and the bundles were bathed in this solution after the preparation for about one hour before use.

The details of the technique used for the *visco-elasticity determinations* have been described earlier (EDMAN 1959 a). Resting fibre bundles were subjected to quick stretch (within 2 sec), and the tension was recorded until a constant level had definitely been reached. The determinations were performed subsequent to a contraction-relaxation cycle when the effect of zinc + ATP was studied. The fibres were equilibrated with the test solution for 10 min before stretch. Control experiments with veronal buffer solution alone were carried out on resting fibre bundles without previous contraction and relaxation.

II. *Zinc analysis experiments*

Determination of dry weight and nitrogen content

The fresh or glycerol-extracted muscle tissue was dried to constant weight at + 105° C. The nitrogen content was determined by means of micro-Kjeldahl technique.

Analysis of zinc in the muscle tissue

For each zinc determination about 20 mg dry tissue was used. The organic matter was destroyed by wet combustion with a mixture of HNO_3 — H_2SO_4 — $HClO_4$. Zinc

was determined colorimetrically (Zeiss "Elko") after extraction with dithizone-carbon-tetrachloride at pH 4.75 according to SANDELL (1950); the addition of sodium thiosulphate was omitted.

Determination of the zinc loss during glycerol-extraction

Immediately after careful exsanguination of the animal the psoas muscle, deprived of its fascia, was removed from the animal and reduced to a pulp in an agate mortar. After this treatment the muscle mass consisted of undifferentiated brei, single fibres and fragments of fibre bundles. Two samples of the muscle pulp were taken for analysis of dry weight and nitrogen and zinc content. From the rest of the pulp two samples (about 1.2 g fresh weight) were each placed in 250 ml of a water solution with pH 7.0 containing 50 per cent glycerol and 10 mM sodium phosphate buffer, *i. e.* the solution used for extraction of the fibre bundles in the contraction-relaxation experiments. The glycerol-extraction was performed in polyethylene flasks at — 20° C and was continued for 21 days.

After the glycerol-extraction the muscle mass was washed twice in magnesium-free veronal buffer solution (about 100 ml) by gentle stirring for 15 min followed by 30 min centrifugation (3,500 r. p. m.). This was followed by determination of dry weight and zinc content.

Determination of the zinc content in the glycerol-extracted muscle tissue after treatment with EDTA and ATP

Muscle bundles previously extracted with glycerol for 4 weeks were pulpified and thereafter washed twice in magnesium-free veronal buffer as just described. Two samples of the muscle mass treated in this way were taken for analysis of zinc and nitrogen. The rest was subjected to further treatment with:

1. 1 mM EDTA for 30 min,
2. 1 mM EDTA for 60 min,
3. veronal buffer solution alone for 30 min,
4. veronal buffer solution alone for 60 min,
5. 1 mM ATP for 30 min.

For each treatment 5 samples of the muscle pulp ($\sim$ 20 mg dry weight each) were studied, and for each muscle sample a bath volume of 500 ml was used. After the incubation the muscle tissue was separated from the bath by filtration, and its dry weight and zinc content were determined. The filter paper used had been previously rinsed with hydrochloric acid and hydrofluoric acid.

Determination of zinc uptake by glycerol-extracted muscle

Glycerol-extracted muscle, after pulpefaction and washing as described above, was incubated for 60 min in magnesium-free veronal buffer solution containing zinc in different, low concentrations. In some experiments, the medium also contained magnesium. According to an earlier study (EDMAN 1959 c) 60 min is sufficient for reaching equilibrium between the zinc taken up by the muscle tissue and the zinc concentration in the bath. A muscle quantity, corresponding to about 20 mg dry weight, was used in each test. The bath volume was 1,000 ml (in a few series 500 ml). Such a large volume was used in order to prevent the concentration of zinc in the bath from decreasing too greatly, in no experiment more than 13 per cent, during the incubation. The different bath solutions were prepared by diluting a 1.00 mM stock solution of $ZnCl_2$ with veronal buffer solution. This latter had been previously analyzed for zinc (usually 0.00020 mmoles Zn per litre). In order to preclude metal contamination the incubation was performed in sealed borosilicate glass flasks. These were gently shaken

during the incubation. The muscle mass was finally separated from the incubation medium by filtration, and its dry weight and nitrogen and zinc content were determined. The uptake of zinc by the tissue represents the amount of zinc bound to the muscle in excess of that present in the tissue after 60 minutes' washing in veronal buffer solution only.

Results

I. *Repeated contraction-relaxation cycles with high visco-elastic compliance of the fibre bundles*

The following experiments demonstrate two ways of producing alternate contraction and relaxation in glycerol-extracted fibre bundles while these are in a "plasticized" condition, *i. e.* in a state of increased visco-elastic compliance. As shown earlier (EDMAN 1959 b) the contraction-inhibition and the relaxation of glycerol-extracted fibres is determined by the free zinc ion concentration in the bath. In the present experiments a constant total zinc concentration was maintained. The free zinc ion concentration was changed in one case by changing the ATP concentration and in the other, at constant ATP concentration, by addition of EDTA, a complexon with still stronger metal-complexing ability than ATP.

Fig. 1 shows the effect on the tension produced by changing the ATP concentration at constant zinc (0.6 mM). A total zinc concentration of 0.6 mM in the bath was found to be the lowest concentration at which complete isometric relaxation could be obtained in the presence of 3 mM ATP and 1 mM magnesium. By increasing the ATP concentration, all other variables being constant, the fibre bundles were made to contract. Under the experimental conditions used here the contraction-inducing effect was maximal when the ATP was raised to about 8 mM.[1] Complete relaxation was again induced when the ATP concentration was decreased to 3 mM. In this way, merely by changing the ATP concentration in the bath repeated contraction-relaxation cycles were produced without any significant decrease in contractility. With the high ATP concentration used the fibre bundles were in a markedly "plasticized" state, *i. e.* their passive visco-elastic resistance was low. This was established by recording the tension development during and following quick stretch of the fibre bundles after 10 minutes' equilibration in the relaxing medium. The visco-elastic effect is illustrated in Fig. 1 also.

Using the stability constants given earlier (see Methods) the free zinc ion concentration for the combination 0.6 mM total zinc, 1 mM magnesium and 3 mM ATP was calculated to be *0.0070 mM*. When the ATP concentration was changed to 7 mM, the concentration of free zinc was decreased to *0.0019 mM*. The corresponding changes in the uptake of zinc by the muscle fibres will be shown under Results II, 2.

[1] That an ATP increase beyond 8 mM, with consequent greater complexing of zinc, did not bring about further contraction was probably due to the fact that, at this concentration, the *contraction*-inducing effect of ATP begins to be "overoptimal" as first described by WEBER (1951, 1954).

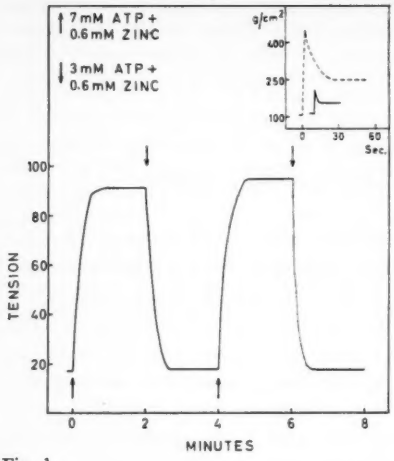

Fig. 1.

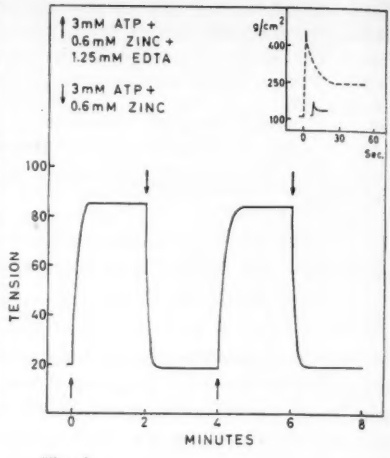

Fig. 2.

Fig. 1. Repeated isometric contraction-relaxation cycles in a glycerol-extracted fibre bundle at constant zinc (0.6 mM) but varied ATP concentration in the bath.

Contraction induced by 7 mM ATP, relaxation by 3 mM ATP.[1] One cycle preceded the two cycles illustrated. Cross section of the fibre bundle: 103 × 178 μ.

Inserted figure: Response to quick stretch of resting fibre bundles equilibrated with veronal buffer solution alone (broken line) and, subsequent to a contraction-relaxation cycle, with 3 mM ATP + 0.6 mM zinc + 1 mM magnesium (solid line). The curves (mean of 5 exp.) arbitrarily displaced along the abscissa.

Fig. 2. Repeated isometric contraction-relaxation cycles in a glycerol-extracted fibre bundle at constant zinc (0.6 mM) and ATP (3 mM) concentrations.

Contraction induced by 1.25 mM EDTA, relaxation by removal of EDTA. One cycle preceded the two cycles illustrated. Cross section of the fibre bundle: 103 × 178 μ.

Inserted figure: Response to quick stretch of resting fibre bundles equilibrated with veronal buffer solution alone (broken line) and, subsequent to a contraction-relaxation cycle, with 3 mM ATP + 0.6 mM zinc + 1 mM magnesium (solid line). The curves (mean of 5 exp.) arbitrarily displaced along the abscissa. Note (cf. Fig. 1) that the increase in visco-elastic compliance produced by 0.6 mM zinc + 3 mM ATP is augmented by the previous treatment of the fibres with EDTA.

Fig. 2 shows repeated contraction-relaxation cycles at constant zinc (0.6 mM), magnesium (1 mM) and ATP (3 mM). On addition of 1.25 mM EDTA the fibre bundle contracted. When EDTA was removed the fibre bundle relaxed again. The contraction-relaxation cycles produced in this way were also fully repeatable. Probably the underlying mechanism was essentially the same as in the former experiment, *i. e.* the reversal from relaxation into contraction was due to a lowering of the free zinc ion concentration in the bath, brought about in this case by addition of EDTA instead of by an increase in the ATP concentration. The free zinc concentration existing in the bath when EDTA was absent was *0.0070 mM* (cf. above). The low visco-elastic resistance of the fibre bundles in the relaxed state is also illustrated in Fig. 2.

[1] At the arrows in this and the next figure the previous bath solution was completely exchanged for the new one.

Table I. Zinc content and dry weight of ground rabbit psoas muscle before and after three weeks' glycerol-extraction

Animal	Zinc content of rabbit psoas (mmoles/kg dry weight)		*Dry weight* of muscle after glycerol-extraction. Per cent of fresh muscle dry weight	*Zinc loss* from muscle during glycerol-extraction. Mmoles/kg dry extracted muscle
	Fresh muscle	After glycerol-extraction		
I	2.08 1.98 *2.03*	1.05 0.94 *1.00*	76.85 74.00 *75.43*	1.66 1.74 *1.70*
II	1.82 1.86 *1.84*	1.16 1.08 *1.12*	69.86 67.27 *68.57*	1.45 1.69 *1.57*
III	1.79 1.88 *1.84*	1.03 1.10 *1.07*	79.03 76.70 *77.87*	1.23 1.35 *1.29*
IV	1.94 2.14 *2.04*	0.98 1.03 *1.01*	66.40 70.63 *68.52*	1.94 2.00 *1.97*
V	1.95 1.94 *1.95*	1.17 1.02 *1.10*	74.61 74.29 *74.45*	1.44 1.59 *1.52*
VI	1.95 1.95 *1.95*	1.00 1.06 *1.03*	67.06 66.32 *66.69*	1.91 1.88 *1.90*
VII	2.10 2.09 *2.10*	1.18 1.15 *1.17*	83.03 82.99 *83.01*	1.35 1.37 *1.36*
VIII	2.11 2.25 *2.18*	1.09 0.98 *1.04*	79.28 79.76 *79.52*	1.57 1.84 *1.71*
Means between the animals....	1.99 ± 0.04	1.07 ± 0.02	74.26 ± 2.08	1.63 ± 0.08

The values to the left in the columns represent single analyses, italicized values to the right are means.

Nitrogen content in fresh muscle: 15.3 per cent, after glycerol-extraction: 14.3 per cent of dry tissue weight, as determined with two samples from each of two rabbits.

II. *Zinc analyses*

In a previous study (EDMAN 1959 c) the uptake of zinc by glycerol-extracted muscle tissue was studied over a large concentration range of zinc in the bath. It was found that the zinc ion had a great affinity for the extracted muscle fibres, and, that the fibres were able to accumulate zinc in great excess of the threshold concentration needed for total relaxing effect. In the present investigation, carried out with microanalysis technique, a separate study was made of the zinc uptake by the extracted fibres at very low zinc concentrations in the bath. On the basis of these determinations the uptake of zinc by the fibres after treatment with different combinations of zinc and ATP may be calculated. In order to see if the zinc amount that must be taken up by the extracted fibres for relaxation could be covered by the supply of zinc in the

Table II. Effect of treatment with EDTA and ATP on zinc content in glycerol-extracted muscle tissue

Treatment of the glycerol-extracted muscle tissue	Time for incubation (minutes)	Zinc content in muscle tissue (mmole/kg dry weight)	Number of experiments
Buffer solution alone	30	0.71 ± 0.06	5
	60	0.68 ± 0.05	5
EDTA, 1 mM	30	0.37 ± 0.06	5
	60	0.35 ± 0.05	5
ATP, 1 mM	30	0.48 ± 0.04	5

Zinc content of the glycerol-extracted muscle tissue immediately before the treatments described in the table: 0.84 ± 0.04 mmole per kilogram dry weight; *nitrogen content:* 11.2 per cent of dry weight of the glycerol-extracted tissue.

living fibre, the amount of zinc removable by glycerol-extraction of fresh muscle and by further treatment with EDTA and ATP has also been determined.

1. *Concentration of zinc in fresh muscle, and after its extraction with glycerol and after treatment with EDTA and ATP*

In a series of experiments two samples of the psoas muscle from eight rabbits were analyzed for dry weight and zinc content before and after three weeks glycerol-extraction and subsequent washing with veronal buffer solution (for details, see Methods II). The results are summarized in Table I. With the weight loss of the fibres (column IV) taken into account, the real zinc loss from the fibres caused by the glycerol-extraction and washing was calculated (column V). The mean value of zinc lost per kilogram of glycerol-extracted muscle tissue was 1.63 ± 0.08 mmoles *i. e.* 60 per cent of the zinc originally present in the fresh muscle. This value is in good agreement with the preliminary estimate of 2 mmoles given earlier (EDMAN 1959 c).

The aim of the experiments presented in Table II was to show whether or not the zinc content in glycerol-extracted and thoroughly washed fibres may be further decreased by treatment with the complexons EDTA and ATP. All experiments were performed with the same original muscle preparation. The zinc content in the preparation used was lowered to 0.70 mmole per kg dry material by simple washing in the veronal buffer solution. By treatment with 1 mM EDTA the zinc content was further decreased to 0.35 mmole per kg. A decrease in the zinc content of the fibres beyond that produced by glycerol-extraction and exhaustive washing with the buffer solution may also be attained by treatment with 1 mM ATP although not as markedly as by

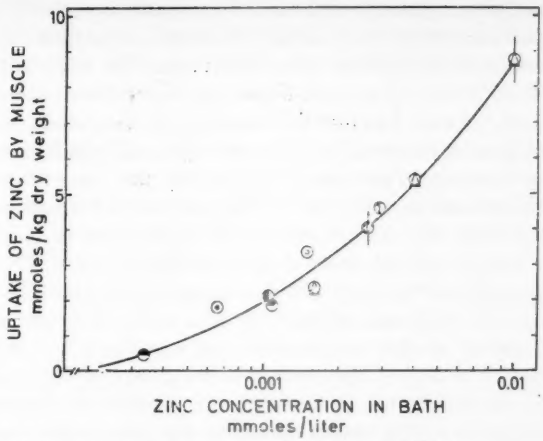

Fig. 3. Uptake of zinc by glycerol-extracted muscle tissue at pH 7.3.
Medium: veronal buffer solution without magnesium except for the experiments symbolized with △, where magnesium was present in a concentration 9.25 times that of zinc. Experiments ○ and △ were performed with the same muscle preparation; the other symbols signify different muscle preparations. Each symbol represents the mean of 4—5 experiments. The standard error of the mean is indicated with a vertical bar if it exceeds the symbol size. Curve fitted by sight.
Nitrogen content: 11.5 ± 0.1 per cent of dry muscle weight.

1 mM EDTA. In the experiments demonstrated in Table II the zinc content was decreased from 0.71 to 0.48 mmole per kg dry material by 30 min treatment with 1 mM ATP.

The experiments do not permit a quantitative evaluation of the difference in the effectiveness of ATP and EDTA in decreasing the zinc content of the muscle fibres, since the ATP concentration continuously decreases during the incubation of the muscle due to enzymatic dephosphorylation. The breakdown product, ADP, is less able than ATP to complex metals (MARTELL and SCHWARZENBACH 1956, WALAAS 1958, WEITZEL and SPEHR 1958). According to earlier data on the ATP-splitting under conditions similar to those in the present experiments (EDMAN 1959 b), about 25—50 per cent of the original amount of ATP in the bath had been dephosphorylated to ADP by the end of the incubation.

The ATP-produced lowering of the zinc content of the glycerol extracted fibres is of particular interest, since, as discussed above and later, ATP may play a part as metal-complexing agent in the contraction-relaxation cycle in the living cell.

2. *Zinc uptake by the glycerol-extracted muscle fibres*

Fig. 3 illustrates the uptake of zinc by glycerol-extracted muscle fibres equilibrated with magnesium-free veronal buffer solution (pH 7.3) containing zinc in different, low concentrations. Without addition of zinc, the buffer solu-

tion contained about 0.00020 mM zinc. The uptake is therefore given as the amount of zinc taken up in excess of the zinc present in the fibres after equilibration with veronal buffer solution alone. With some zinc concentrations of particular interest in the contraction-relaxation experiments presented in this work the uptake of zinc was also determined with magnesium present in the bath. The magnesium concentrations were *9.25* times higher than the respective zinc ion concentrations tested, *i. e.* roughly the same proportion as between free magnesium and free zinc in the contraction-relaxation experiments. As is evident from Fig. 3 the addition of magnesium in the bath did not significantly modify the uptake of zinc by the fibres.

It was demonstrated on page 333 that complete relaxation was produced by 0.6 mM zinc + 1 mM magnesium + 3 mM ATP. The free zinc ion concentration existing at this combination was calculated to be 0.0070 mM, which, according to Fig. 3, corresponds to an uptake of 7.5 mmoles zinc per kilogram dry extracted fibre tissue. With the combination 0.6 mM zinc + 1 mM magnesium + 7 mM ATP, by which the fibres were converted from relaxation into strong contraction (free zinc ion concentration: 0.0019 mM) the uptake is found to be about 3.5 mmoles per kilogram dry tissue. A difference in the zinc uptake of around 4 mmoles per kilogram thus seems to be sufficient to produce a change from marked contraction to total relaxation. It may be noted in this connection that complete isometric relaxation has been found to be produced by a concentration of free zinc in the bath as low as 0.0025 mM (EDMAN 1959 b), if magnesium is absent and the ATP concentration is the lowest possible (0.04—0.08 mM), corresponding to an uptake of 4.0 mmoles per kg dry tissue.

Thus, the total uptake of zinc by the extracted fibres needed for complete isometric relaxation of the contractile element seems to be about twice as great as the total amount of zinc present in the living muscle. The difference in the zinc uptake by the fibres at complete relaxation and pronounced contraction is also roughly twice as great as the amount of zinc lost from the muscle fibres during glycerol-extraction and subsequent washing. Probably, however, only a fraction of the zinc taken up is bound to functionally significant sites in the fibre protein in view of the fact that the fibres are able to bind zinc in great excess of the concentration necessary for complete relaxation. The functionally relevant amount of zinc needed for the relaxing effect may therefore be assumed to be entirely covered by the total zinc supply in living muscle.

Discussion

On the basis of studies of the contractile element in isolated systems it is generally accepted that ATP supplies energy for the contraction process in muscle in vivo. However, it remains uncertain if ATP is the immediate source of energy for the contractile process in the living cell, as is probably the case

in isolated contractile systems, or if ATP supplies energy through some intermediate link (WEBER and PORTZEHL 1954, FLECKENSTEIN 1955, MORALES, BOTTS, BLUM and HILL 1955, BUCHTHAL, SVENSMARK and ROSENFALCK 1956, PERRY 1956, GELFAN 1958, CHANCE 1959, DAVIES, CAIN and DELLUVA 1959, GERGELY 1959, JÖBSIS 1959). Moreover, while ATP-induced contraction of glycerol-extracted fibres is always associated with dephosphorylation (WEBER and PORTZEHL 1954, WEBER 1955), there is still disagreement on whether or not there is dephosphorylation during contraction in living muscle (MUNCH-PETERSEN 1953, FLECKENSTEIN 1955, MOMMAERTS 1955, DIXON and SACKS 1958, SACKS 1959, CHANCE 1959, DAVIES, CAIN and DELLUVA 1959, GERGELY 1959, JÖBSIS 1959). This uncertainty as to the function of ATP in the contraction-relaxation process still limits our possibilities for physiological evaluation of results obtained in studies of the reaction between ATP and glycerol-extracted muscle fibres.

Several factors have recently been described (for earlier ref. see EDMAN 1959 d; WATANABE and SLEATOR 1958 and EBASHI and EBASHI 1959), which are able in the presence of ATP to induce relaxation of the contractile element. The greatest interest has been focused on the relaxing protein factor, first described by MARSH (1952) and remarkable as it is a physiological constituent in skeletal muscle. No studies of the interaction of the zinc ion and protein factor activity have yet been presented. Cadmium, which is a closely related metal but, in contrast to zinc, unphysiological to muscle, inhibits the protein factor even more than calcium does (EBASHI *et al.* 1956). In any case, the many differences existing between zinc relaxation and protein factor relaxation, *e. g.* with respect to interaction of magnesium, calcium, ATP and other nucleotides, make it probable that zinc is not dependent on protein factor activity for its own relaxing effect (EDMAN 1959 d).

The comparatively high concentration of zinc in living muscle invites speculation about the possibility of a direct physiological role for zinc as a relaxing factor in muscle. The results in the present work show that the amount of zinc necessary for inducing relaxation of the contractile element in vitro may be covered by the total amount of zinc in the living muscle cell.

What would then be a plausible mechanism for the action of zinc as relaxing factor in muscle? In the glycerol-extracted fibre the effect of zinc, as pointed out earlier (EDMAN 1959 d), is inhibition of the contractility by blocking of functional sites in the contractile element, thereby permitting ATP or some other nucleoside triphosphate to bring about relaxation, and, if the nucleotide concentration is high, simultaneous plasticizing of the fibres. A similar mechanism in the living cell would require zinc to unblock and reblock the strategic sites in the contractile element alternately to permit contraction and relaxation, respectively. This could be achieved by release of a substance with great zinc-complexing ability into the appropriate compartment of the fibre at the moment of excitation. The functional sites in the contractile element would

thereby be freed from zinc. The complexed zinc should be returned again at the end of the contraction phase, however, for reblocking of the functional sites — made possible, for instance, by breakdown of the complexant. The validity of such a mechanism for production of repeated contraction-relaxation cycles with zinc in glycerol-extracted fibres is evident from the experiments shown in Fig. 1 and Fig. 2.

In view of the great ability of ATP to complex zinc, one is inclined to assume that ATP would play an essential part in the regulation of zinc as relaxing factor in living muscle. The simplest assumption would be that ATP functions as contraction-inducing agent, the interaction with zinc occurring parallel to the reaction between ATP and the contractile element. There are altogether about 5 mmoles ATP per kg skeletal muscle (BERGKVIST and DEUTSCH 1954, see also WEBER and PORTZEHL 1954), but it is conceivable that only part of this ATP could be immediately accessible to the contractile element. Some ATP may be stored, *e. g.* in the mitochondria, to be released only by a certain incitement. Thus, in accordance with the experiment in Fig. 1, the muscle cell could be assumed to be relaxed at a certain concentration of ATP around the myofibrils when contractility and ATPase activity are inhibited due to blocking of functional sites in the contractile element by zinc. At the moment of excitation a sufficient portion of ATP might be released. Part of the zinc bound to the contractile element would be complexed by the added ATP; contractility and ATPase activity would reappear, and the muscle fibre would contract. By the splitting of ATP the complexed zinc is returned to its original place in the contractile element and the muscle relaxes again, since the ability of the breakdown products of ATP to complex metals is markedly lower than that of ATP itself (MARTELL and SCHWARZENBACH 1956, WALAAS 1958, WEITZEL and SPEHR 1958). In this way ATP would be able to restore the contractility in the cell for just that interval required for the reaction between ATP and the contractile element.

The physiologically relevant complexant of zinc released into the appropriate compartment of the fibre at the moment of excitation might turn out to be a substance other than ATP. At any rate, a mechanism of this kind, an adjustment of the zinc-induced inhibition of the contractile element brought about by a reactant in the contraction process, would seem plausible.

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Circulatory Data in Normal Subjects at Rest and During Exercise in Recumbent Position, with Special Reference to the Stroke Volume at Different Work Intensities

By

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Abstract

Holmgren, A., B. Jonsson and T. Sjöstrand. *Circulatory data in normal subjects at rest and during exercise in recumbent position, with special reference to the stroke volume at different work intensities.* Acta physiol. scand. 1960. *49*. 343—363. — Right heart catheterization has been performed in 18 healthy subjects, 14 males and 4 females, including estimation of cardio-vascular pressures and cardiac output at rest and during different intensities of exercise in recumbent position. During exercise the cardiac output increased linearly with the oxygen uptake. The increase of the arterio-venous oxygen difference in relation to the work load or the pulse rate was higher in the males than in the females. With change from rest to work the stroke volume increased slightly but significantly in the males. By increasing work load it successively decreased to the size at rest. There was a high correlation between the stroke volume on one hand and the work performed at pulse rate 170, the heart volume, and the blood volume on the other. The mean pressure in the pulmonary artery was 5 mm Hg higher during exercise than at rest but was constant at different work loads. The end-diastolic pressure in the right ventricle and the mean pulmonary venous pressure (PCV-pressure) did not change significantly with the increase in pulse rate.

The introduction of cardiac catheterization has greatly increased the possibilities for analysis of the central circulation in patients with different kinds of heart disease. It has been difficult however, for obvious reasons, to obtain

comparative values for healthy individuals through application of this technique. Nevertheless, some results have been published from studies in healthy subjects at rest (STEAD *et al.* 1945, FOWLER, WESTCOTT and SCOTT 1953, BARRAT-BOYES and WOOD 1957 and KJELLBERG *et al.* 1959) and also during exercise (RILEY *et al.* 1948, DEXTER *et al.* 1951, SLONIM *et al.* 1954, DONALD *et al.* 1955, FREEDMAN *et al.* 1955, VARNAUSKAS 1955, HOLMGREN *et al.* 1957 and MUSSHOFF *et al.* 1959). As these normal series are relatively small and since somewhat different techniques of cardiac catheterization have been used, it is of interest that results obtained on additional normal material be reported from different groups working in this field.

In the studies published from this laboratory on the hemodynamic conditions observed in different series of cardiac patients (HOLMGREN *et al.* 1957, JONSSON, LINDERHOLM and PINARDI 1957, HOLMGREN *et al.* 1958, HOLMGREN, KARLBERG and PERNOW 1959) particular attention has been paid to the conditions during exercise at different work intensities. This applies also to questions concerning which factors limit the physical working capacity. This has resulted in the collection of a comparatively extensive material illustrating the circulatory adjustment taking place during exercise in cases of different kinds of circulatory impairment. This material has been supplemented from time to time with healthy individuals.

Circulatory data obtained in normal subjects during cardiac catheterization can also be expected to throw light upon physiologic problems earlier inaccessible to analysis under relatively normal physiologic conditions and in man. This is especially true of the behaviour of the stroke volume with variations in the cardiac output. Thus it has been regarded more or less as axiomatic that the cardiac output, during physical work for example, is increased by an increase in both stroke volume and heart rate. It has been assumed that an increase in the cardiac output is conditional upon a displacement of blood from the systemic circulation to the heart, and, according to Starling's law, this would be accompanied by an increase in the stroke volume. Earlier experiments with Grollman's acetylene method on man have also indicated that this is true (CHRISTENSEN 1937). According to the results of these experiments the stroke volume of the heart increases up to three times during exercise and largely linearly with the increase in the oxygen uptake and the pulse rate. The majority of investigations during recent years, however, have not supported this view, apparently regardless of the method used for determination of the cardiac output, as shown by reviews of accessible data by SJÖSTRAND (1956) and RUSHMER and SMITH (1959). According to these collocations the stroke volume seems to remain practically constant during exercise and the same as at rest. Thus the cardiac output would increase during exercise almost exclusively as a result of an increase in the heart rate. On the other hand, these observations are contradicted by results obtained by MUSSHOFF *et al.* (1959) and MITCHELL *et al.* (1958), who reported an increase in the stroke volume

during exercise. According to the firstmentioned authors, this amounted to about 50 per cent. MITCHELL *et al.* (1958) studied the circulatory accommodation in upright position but the investigations included in the reviews mentioned were done in recumbent position. This may explain the different results. It is naturally of fundamental importance for our understanding of the general regulation of the blood circulation that further light be thrown on this question.

Both the desirability of a report on circulatory data from normal individuals during cardiac catheterization and the need for additional data to clarify the problem of the stroke volume during exercise have been regarded as motives for publication of the present investigation.

Material

Eighteen individuals have been studied. They have been divided in three groups. In group A (no. A: 1—6) are six male healthy volunteers (blood donors registrated at the hospital blood bank). In group B (no. B: 1—8) are eight male and in group C (no. C: 1—4) four female patients who were referred to the medical department at the hospital because of systolic murmur. In case no. C: 4 a wide main trunk of the pulmonary artery had been observed at mass radiography.

None of these patients had any heart symptoms and they were all also in other respects subjectively healthy. The murmur had been detected at routine examinations. At examination in the medical department it was found to be an early systolic murmur, grade 1—2, over the pulmonary area. The phonocardiogram showed that the murmur was short, early systolic, and of low frequency. The second sound was not abnormally split. The roentgenological examination showed in every case a heart of normal size and configuration. The electrocardiograms were normal both at rest and during exercise. The sedimentation rate and antistreptolysin titres were normal. In all patients the murmur was classified as a physiological murmur.

The only heart diseases giving the same type of murmur are an atrial septal defect (including partial abnormal venous return) and a very mild pulmonary stenosis with a pressure gradient in the order of 10—15 mm Hg. However, an atrial septal defect of any functional importance will result in an enlargement of the heart (JONSSON *et al.* 1957). Furthermore, at heart catheterization no left to right shunt and no significant pressure gradient over the pulmonary area was found. The patient with a wide pulmonary arterial trunk was also normal as the physical and roentgenological examination could exclude a pulmonary stenosis, a pulmonary hypertension and a left to right shunt. This was confirmed at heart catheterization. For the purpose of this study all these patients can therefore be regarded as normals.

Six of these patients have earlier been reported as controls in a study dealing with the importance of the adjustment of the peripheral blood flow for the physical working capacity (HOLMGREN *et al.* 1957). Another case used as a control in this previous report is not included in the present study because he had heart symptoms.

Methods

Investigation procedure. The healthy volunteers (case A: 1—A: 6) were catheterized after one week's thorough investigations with the methods described below. The catheterization was performed in the morning and the subject was allowed to leave the hos-

Table I. Some anthropometric data of 18 healthy subjects

Case no.	Sex	Age, year	Height, cm	Weight, kg	B. S. A. m^2	Heart vol. ml.	THb. g	THb. g/kg body weight	Blood vol. L	Working capacity			
										Sitting		Supine kpm/min.	Supine during cath. kpm/min.
										kpm/min.	Per cent of predicted		
A:1	M	28	182	77.6	2.00	940	923	1.19	6.4	1,100	71	930	880
A:2	M	40	181	73.8	1.95	855	796	1.08	6.4	1,300	109	1,150	1,200
A:3	M	23	175	69.5	1.86	900	904	1.30	6.4	1,200	87	1,150	1,100
A:4	M	20	171	69.5	1.82	935	827	1.19	6.2	1,200	97	1,060	1,160
A:5	M	22	171	70.0	1.83	975	893	1.28	5.8	1,050	78	1,140	1,140
A:6	M	22	179	61.0	1.78	860	780	1.28	5.6	1,200	104	1,300	1,260
B:1	M	16	180	64.3	1.85	660	685	1.07	5.1	940	97	1,240	900
B:2	M	17	170	55.0	1.64	770	635	1.15	4.5	920	105	1,160	750
B:3	M	20	170	68.0	1.80	910	745	1.09	4.7	1,200	111	1,200	1,180
B:4	M	16	168	44.6	1.49	580	455	1.02	3.3	660	118	—	610
B:5	M	20	176	72.5	1.86	1,135	915	1.26	6.9	1,200	86	1,200	1,250
B:6	M	19	175	71.5	1.86	820	700	0.98	5.0	900	90	—	920
B:7	M	19	173	55.0	1.66	780	600	1.09	4.0	1,000	123	1,140	1,000
B:8	M	23	179	65.0	1.86	680	730	1.12	5.0	900	81	1,000	900
C:1	F	16	163	77.2	1.82	690	510	0.66	4.4	900	138	—	870
C:2	F	17	174	62.0	1.76	655	565	0.91	5.3	600	80	—	570
C:3	F	25	167	60.2	1.66	560	515	0.86	4.7	440	68	—	600
C:4	F	20	158	48.0	1.56	605	470	0.98	4.5	600	103	—	500

pital in the afternoon. The subjects of group B and C were hospitalized for at least 1—2 weeks. The catheterization was usually the last investigation performed. All subjects had been trained to the experimental procedure and were fully informed of the catheterization.

The methods for determination of total amount of hemoglobin (THb), blood volume and heart volume in the prone position have been the same as earlier reported from this laboratory (HOLMGREN *et al.* 1957).

The physical working capacity was determined as the work performed on a bicycle ergometer at pulse rate 170 (PWC_{170}) obtained by extra- or interpolation from the linear pulse rate — work load relation on a diagram using the pulse rate after 6 min work on each load. In this study no correction was made for non steady state of the pulse rate at the highest load.

Right heart catheterization. Right heart catheterization was performed with the patient in the supine position during the whole procedure including the work test. A double lumen catheter was used in all cases to allow simultaneous measurement of pulmonary arterial wedge pressure and pulmonary arterial pressure on one hand and pulmonary arterial and right ventricular on the other. By watching the pressure tracings on the oscilloscope it was possible to change the position of the catheter without using fluoroscopy during the work test. Oxygen uptake at rest and during exercise were measured

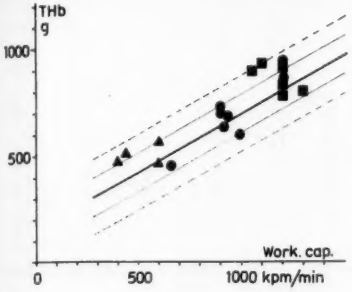

Fig. 1

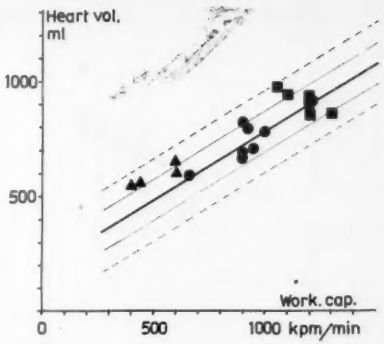

Fig. 2

Fig. 1. The relationship between THb and PWC_{170}. ▲ females, ■ males group A, ● males group B. The straight lines represent the regression line ±one standard error of estimate and the dashed lines ±two standard errors of estimate obtained from determinations on 58 healthy subjects (HOLMGREN *et al.* 1957).

Fig. 2. The relationship between heart volume and PWC_{170}. Lines and symbols, see Fig. 1.

by the Douglas bag technique. During exercise the sampling for estimation of the cardiac output was made between the 4th and 8th minute of work on each load. Blood pressures were recorded by the Svema-Elema strain-gauge mechano-electrical transducer and recorded with an Elema Ecg-apparatus "Klinik" (HOLMGREN 1956). The reference for zero pressure in the supine position was taken as 5 cm below the insertion of the fourth rib at the sternum. Mean pressures were obtained by means of electric integration.

Blood gas samples. Blood samples were analyzed for O_2 and CO_2 content and O_2 capacity with the Van Slyke technique in part of the material (Case B: 3, B: 4, B: 6, B: 7, B: 8, C: 2, C: 3, C: 4) and spectrophotometrically in the rest (HOLMGREN and PERNOW 1959). Oxygen saturation, when analyzed with Van Slyke technique was corrected for physically dissolved oxygen but not for inactive hemoglobin and carboxyhemoglobin. The normal value for the arterial blood was lower than that obtained with spectrophotometric technique. The methodological error involved in the determination of cardiac output at rest and during moderate muscular work has earlier been reported from this laboratory (HOLMGREN and PERNOW 1959 and 1960). Expressed as the error of a single determination, it amounted to 8.2 and 5.2 per cent respectively.

Results

Physical working capacity in sitting position was 1,175 kpm/min in group A (range 1,050—1,300), 965 kpm/min in group B (range 660—1,200) and 623 kpm/min (range 440—900) in group C. All except one case were in steady state with regard to the pulse rate at the highest load. The PWC_{170} "sitting" was normal when related to THb and heart volume in the prone position (HOLMGREN *et al.* 1957), in all subjects (Table I and Fig. 1 and 2).

The PWC_{170} supine during catheterization was 1,123 kpm/min (range 880—

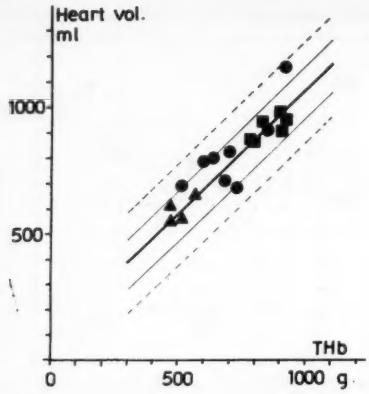

Fig. 3. The relationship between heart volume and THb.
Lines and symbols, see Fig. 1.

1,260) in group A, 939 kpm/min (range 500—870) in group B and 635 kpm/min (range 500—870) in group C. The mean difference between PWC_{170} sitting and PWC_{170} supine during catheterization was 28 kpm/min which is not significant. The scatter was large, however. In subject A: 1, A: 2, A: 3, B: 2 and C: 4 a decrease of the PWC_{170} of 100 kpm/min or more was observed during catheterization. In subject C: 3 an increase of 160 kpm/min was found.

The PWC_{170} supine without heart catheterization was determined in all subjects belonging to group A and in six in group B (B: 1, B: 2, B: 3, B: 5, B: 7 and B: 8). The mean value was 1,121 kpm/min (range 930—1,300) in group A and 1,156 ($n = 6$) (range 1,000—1,240) in group B. The PWC_{170} supine was on an average 40 kpm/min larger than PWC_{170} sitting. The difference was not significant (S. D. of the differences 160 kpm/min). The PWC_{170} supine was on an average 88 kpm/min larger than the PWC_{170} during catheterization. This difference was not significant (S. D. of the differences = 146 kpm/min).

The total amount of hemoglobin (THb) was 1.22 (1.08—1.30) per cent of the body weight (mean and range) in group A, 1.10 (0.98—1.15) in group B, and 0.85 (0.66—0.98) in group C. These values do not differ significantly from the normal values presented earlier (HOLMGREN *et al.* 1957).

The hemoglobin concentration was 13.95 g/100 ml (range 12.5—15.4) in group A, 14.29 g/100 ml (range 13.3—15.9) in group B and 12.05 g/100 ml (range 11.0—13.9) in group C.

The blood volume was 87.0 ml per kg body weight (range 83—92) in group A and 77.3 (73—95) and 78.2 (57—93) ml per kg body weight in group B and C respectively. The mean values for group A correspond to those found in young athletes (HOLMGREN 1956). The values for group B and C do not deviate significantly from those found in ordinarily trained young men and women (HOLMGREN *et al.* 1958).

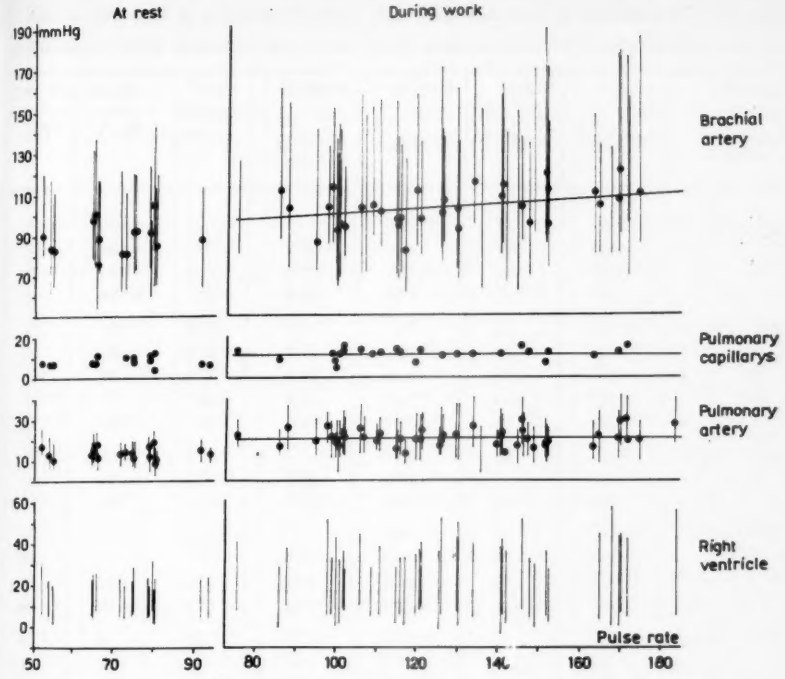

Fig. 4. The relationship at rest and during work between the pulse rate and the systolic, diastolic and mean pressures of the individual cases in respectively brachial and pulmonary arteries, PCV position and the right ventricle. The dots indicate the mean pressure and the vertical lines the pulse amplitude in the individual observation. The regression lines for the mean pressures of the brachial and pulmonary arteries and the PCV-position are drawn.

The heart volume in the prone position was 918 ml (range 855—975) in group A, 792 ml (580—1,135) in group B and 627 (560—690) in group C. The individual values varied within twice the standard error of estimate in relation to THb (HOLMGREN *et al.* 1957) (Fig. 3).

The electrocardiogram at rest, in the standing position and during work was normal in all subjects.

Right heart catheterization. All investigations were carried out without complications.

The pulse rate at rest was 68.3 beats per minute (range 66—79) in group A, 70.5 (range 52—92) in group B and 82.0 (range 74—94) in group C.

The oxygen uptake at rest was 22.8 per cent (range —2 — +43) higher than basal oxygen uptake predicted from sex, age, body length and body weight

Table II. Data obtained in connection with right heart catheterization in 18 healthy subjects

Br. A = brachial artery. PA = pulmonary artery. RV = right ventricle. PCV = pulmonary

Case No. Catheterization no.	Work load kpm/min	Pulse rate beats/min	Oxygen uptake ml/min	Mechanical efficiency per cent	O_2-capacity ml/100 ml	O_2-sat. per cent	
						Br. A	PA
A:1 125/58	Rest	79	290		20.0	95	76
	300	109	975	20.8	20.7	95	59
	600	134	1,543	22.8	21.4	96	53
	900	172	2,270	23.9	21.1	94	45
A:2 126/58	Rest	54	242		17.3	98	79
	300	88	924	21.0	17.8	98	55
	600	106	1,402	24.8	17.8	98	44
	900	130	1,998	24.8	18.4	97	37
	1,200	170	2,804	22.4	19.1	96	32
A:3 129/58	Rest	66	355		17.3	96	74
	300	102	1,064	20.2	17.9	94	55
	600	121	1,594	25.5	18.6	95	50
B:7 44/56	Rest	52	236		19.4	94	70
	200	75	658	22.8	19.7	94	53
	400	98	1,026	24.2	19.8	95	43
	600	126	1,396	24.7	20.5	94	40
B:8 59/56	Rest	72	281		19.2	94	76
	300	107	870	24.3	19.5	94	61
	600	145	1,325	27.5	20.0	94	52
	900	172	1,887	26.8	20.6	94	42
C:1 47/57	Rest	75	256		15.0	97	78
	300	110	894	22.4	15.5	98	54
	600	141	1,289	27.7	16.5	95	47
C:2 12/54	Rest	80	222		15.3	94	73
	200	100	564	29.6	15.6	94	60
	400	136	908	28.0	16.0	94	54
C:3 5/54	Rest	94	224		16.3	94	75
	200	110	556	28.9			
	400	140	889	28.6			
	Rest	80	203		17.9	94	79
	150	117	532	21.8	17.4	95	58
	300	142	755	16.4	17.7	94	51
	900	170	2,012	25.4	18.7	101	42
B:2 42/57	Rest	73	225		17.5	100	82
	300	115	873	22.0	17.9	98	62
	600	152	1,501	22.5	18.6	98	51
B:3 27/55	Rest	65	293		19.4	94	76
	300	86	919	22.8	19.6	97	58

capillary venous. S = systolic. D = diastolic. De = end-diastolic. M = mean

AV-O_2-diff ml/l	Card. outp. l/min	Stroke vol. ml	Pressures, mm Hg				Pulm. vasc. resist. index
			RV S-De	PA S-D-M	PCV M	Br. A S-D-M	
41.4	7.0	90	24—5	23—10—17	11	124—75—92	1.71
82.4	11.8	108	28—5	21— —	11	151—90—104	—
96.8	15.9	119	40—7	40—16—26	11	170—94—115	1.88
101.2	22.4	130	41—6	40—19—29	15	175—95	1.25
37.2	6.6	121	23—5	22— 7—13	7	118—62—84	1.76
81.5	11.3	128	37—10	37—15—26		154—74—103	
95.3	14.7	139	44—10	37—13—25	13	158—71—103	1.60
112.8	17.7	136	42—7	37—10—22	11	160—73—102	1.21
122.6	22.9	135	42—0	32—10—21	12	177—80—106	0.77
36.4	9.9	149	27—8	25—12—18	11	117—54—76	1.35
69.5	15.3	150	36—9	34—13—23	13		1.22
88.2	18.1	150	37—8	31—10—20			
47.3	4.9	96	31—6	26—14—17	8	119—80—90	3.00
83.3	7.9	105	41—8	30—17—22	13	123—81	1.88
96.3	10.6	109	51—7	33—18—26		132—85—103	
113.2	12.3	99	52—6	31—16—21	10	137—86—106	1.49
34.3	8.1	114	24—5	20— 8—13		123—63—81	
67.2	12.9	121		30—13—21		148—71	
87.2	15.1	105		24— 8—17		149—62	
110.6	17.0	99		19		155—69	
29.5	8.8	117	23—5	22— 7—14	8	121—72—92	1.25
69.9	12.8	116	32—2	29—11—17	11	141—69—95	0.86
81.0	15.9	113	38—3	34—12—21	11	157—80—108	1.15
34.0	6.5	81	18—3	13— 3— 8	4	113—63	1.08
54.0	10.1	100		21— 4	5	134—65	
65.0	13.9	103		21— 2		150—63	
32.0	7.0	75	25—5	17— 9—13	7		1.43
59.0	9.6	87		27—15—19			
77.0	11.5	82		24—12—17			
29.0	7.0	88	24—1	15— 8—11		120—67—86	
65.0	8.1	70	33—1	19— 8—13		126—61—82	
79.0	9.5	68	36—2	13		139—67	
115.3	17.5	103	43—7	40—16—28		178—90—120	
33.2	6.8	94	21—5	18—11—14	10	112—63—81	2.68
71.0	12.3	107	28—2	24—10—16	13	137—69—94	0.40
88.5	17.0	112	32—2	25—12—18	12	144—73—94	0.58
37.5	7.8	120	26—4	24—11—15	7	137—76—100	1.86
77.1	11.9	139	28—0	25—12—17	9	161—88—112	1.21

Table II. (cont.)

Case No. Catheterization no.	Work load kpm/min	Pulse rate beats/min	Oxygen uptake ml/min	Mechanical efficiency per cent	O_2-capacity ml/100 ml	O_2-sat. per cent	
						Br. A	PA
	600	126	1,527	23.2	19.9	98	—
	900	148	2,007	25.1	20.1	98	38
B:4 23/56	Rest	92	229		15.8	95	81
	200	116	682	21.2	16.0	96	60
	400	148	1,021	18.7	16.8	95	50
B:5 42/56	Rest	55	267		17.6	94	75
	300	100	924	21.8	18.1	94	64
	600	126	1,454	24.2	18.5	94	52
	900	164	2,136	23.0	19.3	96	45
B:6 42/56	Rest	75	295		18.5	95	70
	300	100	930	22.6	18.8	95	50
	600	130	1,485	24.1	18.6	95	40
	900	168	2,201	22.6	19.8	91	28
	900	146	2,225	23.0	19.3	95	46
	1,200	184	2,983	21.8	19.9	96	34
A:4 132/58	Rest	65	267		19.8	95	78
	300	99	1,006	19.4	20.3	98	62
	600	120	1,558	22.2	20.7	99	52
	900	152	2,157	22.7	21.4	97	46
A:5 135/38	Rest	66	325		17.8	98	79
	300	95	1,043	19.8	18.5	98	61
	600	115	1,585	22.8	19.9	99	52
	900	152	2,016	25.4	19.3	99	43
	1,200	175	2,819	23.1	19.8	95	36
A:6 68/57	Rest	79	302		18.3	99	80
	300	102	1,216	15.7	18.7	99	57
	600	121	1,465	24.7	18.9	99	49
	900	146	2,105	23.8	19.4	98	42
	1,200	165	2,931	21.8	20.0	97	31
B:1 92/57	Rest	80	320		18.0	102	81
	300	111	946	22.8	17.9	101	61
	600	141	1,294	29.4	18.3	101	50

(HARRIS and BENEDICT 1919) in group A, 11.8 per cent (range +6 — +21) in group B and 8.5 per cent (range +5 — +11) in group C.

The oxygen uptake during work in connection with heart catheterization increased ordinarily with increasing working intensity. The mechanical efficiency rose to a mean value of 23.6 per cent during working intensities increasing the pulse rate to above 140 beats per minute.

AV-O_2-diff. ml/l	Card. outp. l/min	Stroke vol. ml	Pressures, mm Hg				Pulm. vasc. resist. index
			RV S-De	PA S-D-M	PCV M	Br. A S-D-M	
99.5	15.3	129	35—1	25—13—17		171—83—	
122.0	16.4	111	30—0	26— 8—15		169—90—	
25.8	8.9	97	24—5	21—10—15	7	114—65—88	1.33
58.5	11.5	99	33—6	28—12—20	12	133—72—98	1.04
77.0	13.8	90	33—4	28—12—20	12	133—70—95	0.90
36.7	7.3	132	21—2	19— 7—10	7	111—65—83	0.75
56.9	16.2	162	36	33—11—18	8	137—76	1.15
79.3	18.3	145	38	35—12—19		140—77—100	
102.2	20.9	127	33	28—10—16	10	147—80—110	0.54
46.2	6.2	83	29—6	20— 7—12	10	120—73—92	0.59
85.5	10.8	109	43—0	29—16		129—68—92	
104.0	14.2	110	50—0	37—10		135—68—92	
125.8	17.5	104	57—0			131—79	
99.7	22.2	152	52—8	40—18—29			
126.1	23.7	129	55—6	40—19—27			
35.2	7.6	117	23—6	19— 8—13	7	133—78—98	1.46
76.8	13.1	132	33—6	29—11—21	11	151—86—113	1.39
100.2	15.6	115	34—6	32—10—20	7	157—84—111	1.51
112.8	19.1	126	36—5	29—11—17	7	189—89—119	0.95
34.8	9.4	143		14— 6—11		118—71—89	
71.0	14.7	155		30—12—19		141—73—86	
101.9	15.6	136		29— 9—15		154—70—97	
111.4	18.1	119		27— 9—18		170—85—111	
116.9	24.1	137		28— 9—19		185—85—109	
39.8	7.7	97	22—5	18— 5—12	8	97—60—74	0.93
80.5	15.1	148	27—8	30—12—21	15	123—80—93	0.71
98.1	14.9	123	40—9	32—14—24	13	133—80—97	1.31
109.5	19.2	132		33—11—24	15	133—87—103	0.83
133.5	22.0	133	44—4	33— 9—22		133—80—103	
43.0	7.6	95	32—9	25—11—19	12	143—83—105	1.71
82.5	11.5	103	38—6	30—14—23	12	155—85—110	1.77
96.6	13.4	95	42—6	32—14—23		162—85—113	

Intracardiac and intravascular pressures (Table II and Fig. 4.) The systolic pressure in the right ventricle was on an average 24.5 mm Hg (range 18—32, n = 17). Corresponding figures for the end-diastolic pressure were 5.0 mm Hg (range 1—9, n = 17). During exercise the systolic pressure increased with increasing load, while the end-diastolic pressure decreased on an average 2 mm Hg.

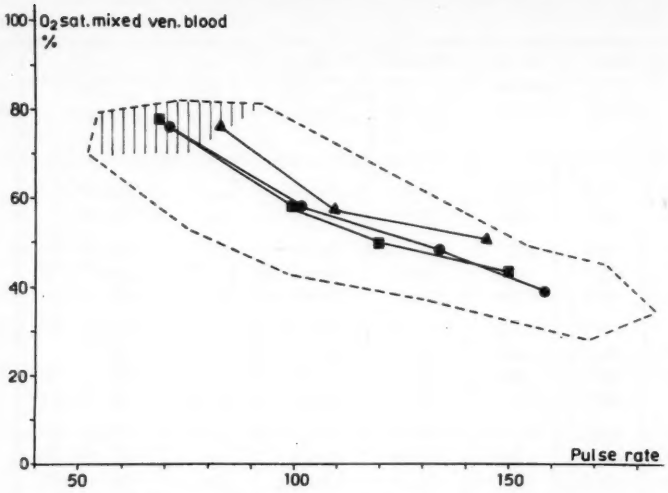

Fig. 5. The relationship between O_2-saturation of the blood in pulmonary artery and pulse rate at rest and during work for the three groups.
Symbols, see Fig. 1.
The area within the dashed line indicates the scatter of the individual values and the shaded area the variations at rest.

The systolic pressure in the pulmonary artery at rest was 20.0 mm Hg (range 13—26). Corresponding figures for mean and diastolic pressures were 13.6 (range 10—18) and 8.6 (range 3—14) mm Hg respectively. The mean pressure in the pulmonary artery during exercise can be expressed by a regression equation $y = 19.2 - 0.01\ x$ (y = mean pressure in pulmonary artery in mm Hg and x = pulse rate). Thus the mean pulmonary arterial pressure remained constant during muscular work with increasing load.

However, the systolic pressure rose in individual cases (A: 3) up to 40 mm Hg. During heavy work significant pressure gradients over the pulmonary valve of a magnitude of up to 15 mm Hg (A: 3) could be observed.

The PCV pressure at rest was measured in 15 subjects and amounted to 8.3 (range 4—13) mm Hg. This individual variation is partly due to differences in the configuration of the thorax.

The pulmonary capillary venous pressure (PCV) was measured during work in eight subjects. The pressure change (y) with increase in pulse rate (x) is expressed by the equation $y = 9.6 + 0.013\ x$. Individual increases of 7 mm Hg (A: 6) were observed.

The pulmonary vascular resistance index expressed as $\frac{\bar{P}_{PA} - \bar{P}_{PCV}}{\dot{Q}/BSA}$ (pulmonary vascular resistance index) was determined at rest in 14 subjects and

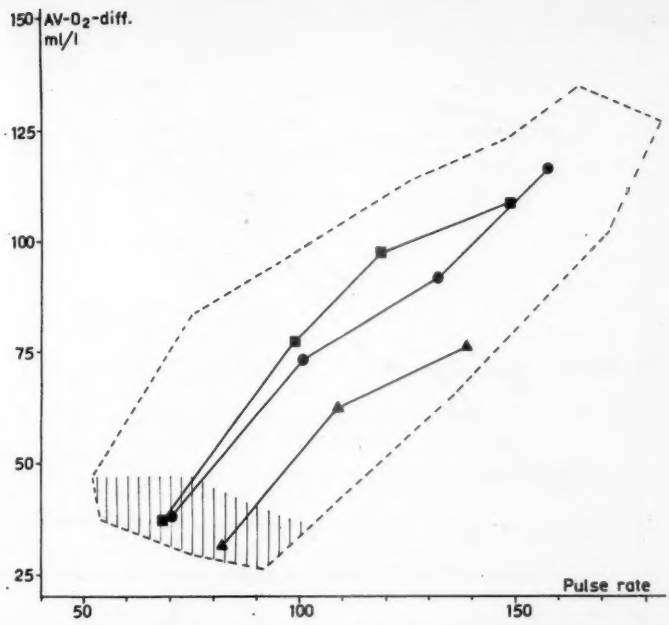

Fig. 6. The relationship between the a-v O_2 difference and pulse rate at rest and during work for the three groups.
Symbols, see Fig. 1.
The area within the dashed line indicates the scatter of the individual values and the shaded area the variations at rest.

amounted to 1.50 units (range 0.59—3.00). During exercise the resistance on the average decreased, especially apparent by high work loads.

The average systolic brachial arterial pressure was 120 (range 111—143) mm Hg at rest. Corresponding values for mean and diastolic pressures were 88.2 (range 81—105) and 68.8 (range 54—83) mm Hg respectively. The arterial blood pressure at rest was consequently well within the range of the normal variation. During exercise the pressures rose with increasing working intensity. The relationship between brachial arterial mean pressure and pulse rate is expressed by the regression equation $y = 86.5 + 0.13\,x$. The increase is less than earlier observed during muscular work in the sitting position (Holmgren 1956). In no subject could abnormally high or low arterial blood pressure be observed during heavy exercise.

The oxygen saturation of the arterial blood at rest varied within normal ranges. During work the arterial O_2-saturation decreased slightly in some cases (Holmgren and Linderholm 1958). Simultaneously the O_2-capacity of arterial blood

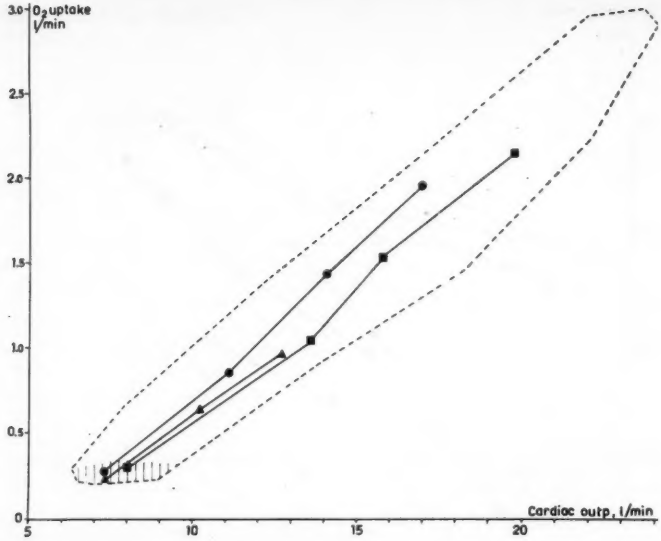

Fig. 7. The relationship between oxygen uptake and cardiac output at rest and during work for the three groups.
Symbols, see Fig. 1.
The area within the dashed line indicates the scatter of the individual values and the shaded area represents the variations at rest.

increased on an average 1.8 vol. per cent in group A, 1.1 vol. per cent in group B and 0.5 vol. per cent in group C.

The oxygen saturation of mixed venous blood (drawn from the pulmonary artery) varied between 70 and 75 per cent at rest. During exercise it decreased with increasing working intensity (Table II and Fig. 5). The lowest values in each individual varied between 28 and 54 per cent. During work the average O_2-saturation was lower in relation to pulse rate in group A and B than in group C.

The arterio-venous oxygen (a-v O_2) difference at rest varied between 25.8 and 47.3 ml/l. During muscular work it increased with increasing working intensity to maximal values between 77.0 and 133.5 ml/l (Table II and Fig. 6). During work the a-v O_2 differences increased to the same degree with increasing pulse rate in group A and B. In group C the average increase of the a-v O_2 difference was lower than in the two male groups.

The cardiac output at rest varied between 6.6 and 9.9 l/min in group A, 5.0 and 8.9 in group B and 6.5—8.8 in group C. The corresponding cardiac indices were 3.38—5.33, 3.01—5.97 and 3.70—4.88 respectively.

During exercise the cardiac output increased to 19.1—24.1 l/min in group A, 12.3—20.9 l/min in group B and 9.5—15.9 l/min in group C. The increase

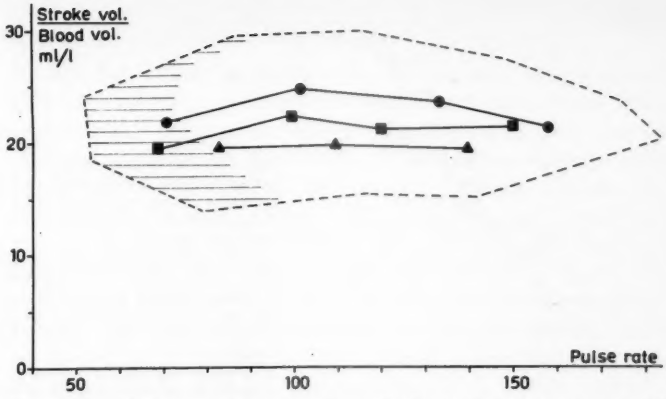

Fig. 8. The relationship between the stroke volume divided by the blood volume and the pulse rate at rest and during work for the three groups.
Symbols, see Fig. 1.
The area within the dashed line indicates the scatter of the individual values and the shaded area represents the variations at rest in male subjects.

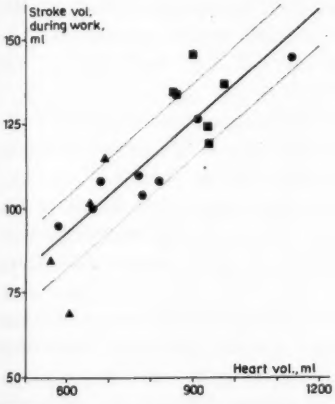

Fig. 9

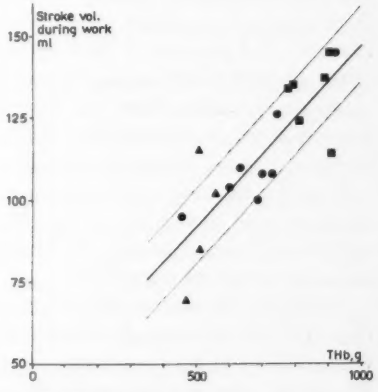

Fig. 10

Fig. 9. The relationship between the stroke volume (mean of the values obtained during work) and the heart volume. The lines represent the regression line ±one standard error of estimate.
Symbols, see Fig. 1. Regression equation: $y = 27.0 + 0.11\ x$, $r = 0.86$. S. D. = 10.9.

Fig. 10. The relationship between the stroke volume (mean of the values obtained during work) and THb.
The lines represent the regression line ± one standard error of estimate.
Symbols, see Fig. 1. Regression equation: $y = 37.2 + 0.11\ x$, $r = 0.84$. S. D. = 11.5

of the cardiac output (Fig. 7) with increasing oxygen uptake did not differ significantly between the three groups.

The stroke volume at rest varied between 90 and 152 ml in group A, 83 and

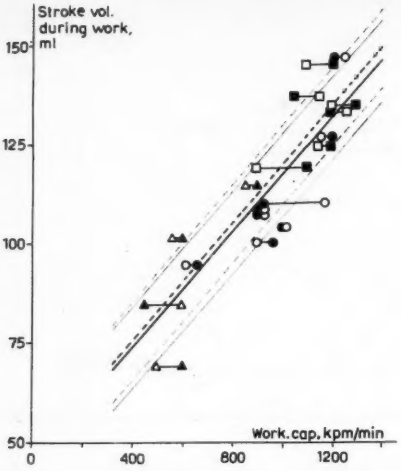

Fig. 11. The relationship between the stroke volume during work and PWC_{170} in sitting (filled symbols) and recumbent position (open symbols).
Symbols, see Fig. 1.
The dashed lines represent the regression line ± standard error of estimate in recumbent position and the straight lines the same in sitting position. Between the symbols of the same subject a line is drawn.
Regression equations: Sitting: $y = 45.2 + 0.072\ x$, $r = 0.87$. S. D. = 10.5; recumbent: $y = 45.5 + 0.074\ x$, $r = 0.88$. S. D. = 10.0.

132 in group B and 75 and 117 ml in group C. The average values for the three groups were 120, 104 and 95 ml. It amounted on an average to 13.1, 13.4 and 14.3 per cent of the heart volume and to 1.92, 2.21 and 1.93 per cent of the blood volume during resting conditions.

On the transition from rest to work there was a significant increase of the stroke volume of 15.6 ml ($P < 0.001$) or 7.88 per cent of the resting value in the male groups. Group C showed no significant changes. (Fig. 8.)

During continued work with increasing load there was a slow return of the stroke volume to the size at rest. If the stroke volume at rest was compared with that obtained during the highest work load in each case no significant difference could be observed.

To study the relationship between stroke volume and dimensional parameters of the circulatory system such as heart volume and total amount of hemoglobin and between stroke volume and PWC_{170} Fig. 9—11 were prepared. To diminish the random variation of the stroke volume due to biological variation a mean value for the stroke volume during exercise was calculated from the observations available. The stroke volume was found to be highly correlated to heart volume and total amount of hemoglobin as well as to PWC_{170}, both in connection with heart catheterization in the supine position as without catheter in the sitting position. Regression equations, correlation coefficients and standard error of estimate are presented in the legends of the figures.

Discussion

The material. For a study involving such procedures as heart catheterization it is hardly possible to get a random selection of the population. There are very few women and the variation in age is small. Only one was over 30 years. It is important to notice that some of the individuals are volunteers and the others are patients, who came to the hospital with the suspicion of heart disease. Throughout the investigation these groups have been treated separately in order to see if any difference could be observed. The volunteers (group A) were blood donors and these could be expected to be above the average with respect to physical training. Most of them practised athletics irregularly. On the other hand the patients (group B and C) can be expected to be less trained. They had no symptoms, but the fact that a heart disease had been suspected, might have influenced their physical activity.

Among the males the dimension of the body (length, weight, surface area) was only slightly higher in group A than in group B. The average age was less in group B and some were very young but all grown up. The dimension of the cardiovascular system, as measured by heart volume and THb, was about 20 % higher in group A. The PWC_{170} showed the same difference, and was in group A of the same order as has been found in Swedish conscripts (LINROTH 1957).

The psychological reaction during heart catheterization is important for the results at rest but probably not to the same extent during exercise. This reaction could possibly be different in group A and B. The volunteer did not have the feeling of being a patient. They were resting only one hour before the catheterization when they came to the hospital and they got no sedation. In group B and C, on the other hand, all felt that each procedure was important to establish the diagnosis. They were hospitalized for at least a week and rested in the morning before the catheterization. Sedation was given. At catheterization no marked difference was found between group A and B. The resting pulse rate and cardiac output was the same. The resting oxygen uptake was slightly higher in group A than in group B which corresponds to the somewhat higher values of BMB usually found in outpatients than in hospitalized patients.

The adaptation of the circulation at rest and during work. The average working capacity was the same in the sitting and supine position, indicating only slight orthostatic effects in the sitting position. To study the effect of the introduction of a heart catheter a similar comparison was performed between the working capacity in the supine position before and during heart catheterization. On an average no significant difference was observed. However, in two cases a large decrease was found during catheterization.

The oxygen saturation of mixed venous blood varied within the same range as in a normal material earlier reported (see HOLMGREN *et al.* 1957). During mus-

cular work with successively increasing load the oxygen saturation decreased, following a curvilinear function (Fig. 5) in the same manner as described earlier. In the male groups the saturation was somewhat lower with increasing intensity than in the female group indicating a higher extraction of oxygen per heart beat.

The a-v oxygen difference at rest also varied within ranges which have been reported earlier in the literature from normal subjects (see HOLMGREN *et al.* 1957). During work the a-v O_2 difference increased according to an approximately linear function reaching maximal values of 134 ml/l in individual cases. The majority of the subjects fall within the border lines of the variation during work presented earlier. It is obvious that the oxygen extraction per heart beat is higher in the male groups than in the female group. This is explained by the fact that the increase of the cardiac output during work (above the resting value) and as a consequence that part of the cardiac output passing the vasculature of the active muscles is much larger in the males than in the females.

The relationship between *the stroke volume at rest* and the blood volume agreed rather well for the three series. On the whole, there was also agreement with the normal values given earlier in the relation between the stroke volume and the heart volume. The variation between the values for different individuals is great, however, which may be explained in part by the rather poor reproducibility in determination of the stroke volume at rest (HOLMGREN and PERNOW 1959).

The stroke volume during work of varying intensity remained largely unchanged. The two male series show about an 8 per cent increase in stroke volume at the transition from rest to exercise, but thereafter the stroke volume decreases successively towards the resting level. If a mean stroke volume during work is computed and correlated with the total amount of hemoglobin and the heart volume, respectively, considerably less variations among the individual observations are obtained than for the stroke volume at rest. This is explained in part by the better reproducibility of the values of the stroke volume during exercise and in part by the fact that the values are mean values from several determinations.

The close relation between the stroke volume and the total amount of hemoglobin (the blood volume) indicates that the stroke volume is regulated to an optimal size in relation to the volume of the vascular system, as suggested by SJÖSTRAND (1957). There is also a linear relation between the heart volume determined with the subject in recumbent position and the blood volume or total amount of hemoglobin (KJELLBERG *et al.* 1949). This can also be interpreted as a result of an active regulation. The heart volume is namely dependent to a great degree of the blood distribution. A direct relationship between the heart volume and blood volume means that the distribution of the blood between the heart, the central vessels and the systemic circulation is largely the same in different individuals in recumbent position (see SJÖSTRAND 1953).

Accordingly the relation between the blood volume and the stroke volume must finally be regarded as the result of a regulatory accommodation of the blood distribution.

As in earlier series of individuals with normal cardiac condition who were examined with the same technique during exercise, the cardiac output increased almost exclusively through an increase in the heart rate. This result differs from that of MITCHELL *et al.* (1958) whose studies showed an appreciable increase in the stroke volume during exercise. In these experiments, however, the resting stroke volume was determined with the subject in upright position. It is to be expected that the posture influences the stroke volume during rest in consequence of the changes of the blood distribution and the heart volume. This has also been demonstrated in investigations of HOLMGREN and OVENFORS (1960) and BEVEGÅRD, HOLMGREN and JONSSON (1960). MITCHELL *et al.* (1958) studied the effect of exercise under only one work load; consequently, their investigations do not provide data on the changes in the stroke volume with varying cardiac output and pulse rate during exercise.

MUSSHOFF *et al.* (1959) observed an increase in the stroke volume of about 50 per cent, on the average, during work, which is considerably greater than in the present investigation and in earlier studies.

Stroke volume and working capacity. The close direct relation observed between the stroke volume measured during exercise in recumbent position and the work performed at a pulse rate of 170 (PWC_{170}) determined in exercise tests with the subject sitting on the bicycle ergometer is remarkable in several respects. It should be emphasized that the PWC_{170} is the same, on the average, in sitting and recumbent positions and with and without the cardiac catheter. There are, however, appreciable individual variations in both directions (Table I). This scatter of the values about the regression line is due to variations in the arterio-venous oxygen difference, in the utilisation efficiency of the bicycle exercise and, especially at smaller work loads, in the basal oxygen consumption. At the same degree of efficiency, the same basal oxygen consumption and the same peripheral utilisation of the blood oxygen, *i. e.* the same degree of adaptation of the peripheral circulation to the working muscles, the oxygen uptake per pulse beat and the work performed at a given pulse rate should vary directly with the stroke volume. The relationship between the stroke volume and the work performed at a pulse rate of 170 in the present material is thus the consequence of the fact mentioned above that the arterio-venous oxygen difference and the utilization efficiency do not vary to any great degree between individuals in a normal material. This is in contrast to the case in patients with the disturbance of the regulation of the peripheral circulation that has been entitled vasoregulatory asthenia (HOLMGREN *et al.* 1957).

The high correlation between the stroke volume and PWC_{170} and that between the stroke volume, the total amount of hemoglobin and the heart

volume respectively explains the linear relation between PWC_{170} on the one hand and the total amount of hemoglobin and the heart volume on the other. The correlation of these values shows the fundamental relations between the size and structure of the vascular system and its function, which, under normal conditions determine the circulatory capacity and thus the capacity for work which, in its turn, is limited by the oxygen uptake.

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Are Dihydroxyphenylalanine Decarboxylase and 5-Hydroxytryptophan Decarboxylase Individual Enzymes?

By

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Abstract

ROSENGREN, E. *Are dihydroxyphenylalanine decarboxylase and 5-hydroxytryptophan decarboxylase individual enzymes?* Acta physiol. scand. 1960. *49.* 364—369. — Using an enzyme preparation from rabbit kidney cortex, the amino acids 3,4-dihydroxyphenylalanine and 5-hydroxytryptophan were found to inhibit each other's enzymatic decarboxylation competitively. Competitive inhibitions of these reactions by m-tyrosine, o-tyrosine, and caffeic acid were also observed. After purification of an enzyme preparation on a column of DEAE cellulose a peak appeared which contained most of the dihydroxyphenylalanine decarboxylase activity present in the extract. In this peak also nearly all the 5-hydroxytryptophan decarboxylase activity was found. The ratio of the two activities was practically the same throughout the peak. The data were in agreement with the assumption that dihydroxyphenylalanine and 5-hydroxytryptophan were decarboxylated by one enzyme.

The presence of a L-3,4-dihydroxyphenylalanine (DOPA) decarboxylase in mammalian tissues was discovered by HOLTZ, HEISE and LÜDTKE in 1938. More recently UDENFRIEND, CLARK and TITUS (1953) found an enzyme catalyzing the decarboxylation of L-5-hydroxytryptophan (5-HTP). It was considered that the decarboxylation of the two amino acids was carried out by two different enzymes (CLARK, WEISSBACH and UDENFRIEND 1954). Since then some doubt has been put forward that 5-HTP decarboxylase is a separate enzyme. Thus in 1958 WESTERMANN, BALZER and KNELL showed that the ratio of the two enzyme activities in a number of peripheral organs of the

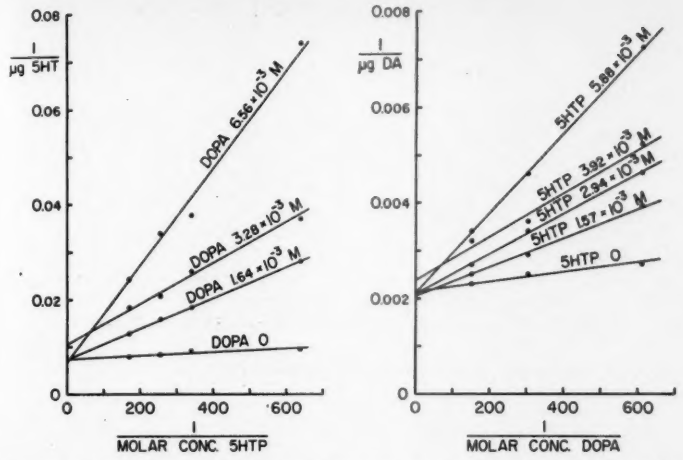

Fig. 1. Rates of dopamine and 5 HT formation from various mixtures of DOPA and 5 HTP
To the left: rate of 5 HT formation from 5 HTP alone and in the presence of various concentrations of DOPA.
To the right: rate of dopamine formation from DOPA alone and in the presence of various concentrations of 5 HTP.
The reciprocal of amine formed in 10 min. are plotted against reciprocal of amino acid concentration. DOPA and 5 HTP concentrations refer to L and DL form, respectively.

rabbit and guinea pig was fairly constant. Furthermore, they found that the formation of 5-hydroxytryptamine (5-HT) from 5-HTP was inhibited by *α*-methyl-DOPA, which was known to inhibit the DOPA decarboxylase. These facts suggested that DOPA decarboxylase and 5-HTP decarboxylase were one and the same enzyme. Independent observations of YUWILER, GELLER and EIDUSON (1959) and of BERTLER and ROSENGREN (1959 a) indicated that 5-HTP and DOPA interact with one enzyme. The problem is studied in further detail in the present investigation.

Experimental

The extract containing the enzyme was prepared from rabbit kidney cortex as described earlier (BERTLER and ROSENGREN 1959 b). For the estimation of enzyme activity 0.5 ml of the extract was put into one side arm of a Warburg cup together with 0.1 mg pyridoxal-5-phosphate in 0.1 ml. In some experiments carried out to determine the Michaelis constants for DOPA and 5-HTP where the substrate and enzyme concentrations were smaller, 0.01 mg pyridoxal-5-phosphate was used. The substrates, dissolved in 1/15 M phosphate buffer, pH 7.5, were placed into the main vessel. The cups were immersed into a water bath at 37° C. A stream of moistened nitrogen was passed through the cups for 10 min. The enzyme was then tipped into the main vessel and was allowed to act for 10 min. The monoamines were extracted with 0.4 N perchloric acid.

Dopamine was determined as described earlier (BERTLER and ROSENGREN 1959 b). 5-HT was estimated after purification on a column containing Amberlite XE 64

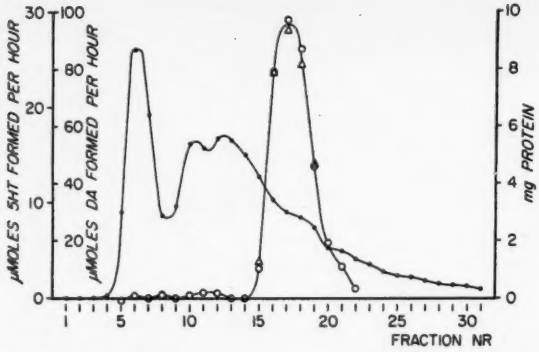

Fig. 2. Fractionation of an enzyme preparation on a DEAE cellulose column.
• mg protein
△ µmoles 5 HT formed
○ µmoles dopamine formed

(BERTLER and ROSENGREN 1959 a). In order to remove the small amounts of 5-HTP retained by the resin, the column was washed with 10 ml 0.02 M phosphate-buffer, pH 6.5, after the extract had passed.

In a first series of experiments the Michaelis constants for DOPA and 5-HTP were determined. The formation of dopamine and 5-HT from various mixtures of DOPA and 5-HTP are given in Fig. 1. In other experiments the amounts of dopamine and 5-HT formed from DOPA and 5-HTP in the presence of the inhibitors m-tyrosine, o-tyrosine and caffeic acid were determined, and the inhibition constants calculated.

Some experiments were performed to investigate if the ratio of the enzyme activities could be varied by purification of the enzyme preparation. For this purpose extracts of rabbit kidney cortex prepared as previously described were centrifuged at 60,000 × *g* for 2 hours in a Spinco centrifuge. The clear supernatant was made 35 per cent saturated with $(NH_4)_2SO_4$ solution, pH 8, and the precipitate was spun down. To the supernatant saturated $(NH_4)_2SO_4$ was added to give 47 per cent saturation. The bulk of the two decarboxylase activities present in the homogenate was found in the latter precipitate. This was dissolved in a few ml of the phosphate buffer and dialysed against redistilled water at 4° C over night. It was then put on a DEAE cellulose column 18 × 20 cm. Gradient elution was carried out principally as described by FAHEY, MCCOY and GAULIAN (1958). The mixing chamber contained 100 ml 0.001 M phosphate buffer, pH 7.5 and the reservoir 0.2 M phosphate buffer of the same pH. The effluent was collected in 7.3 ml fractions. The DOPA decarboxylase activity was determined in the first twenty eluates. In the eluates where this enzyme was found to have a peak the 5-HTP decarboxylase activity was also estimated. Protein was determined as described by LOWRY *et al.* (1951). The results of such an experiment are found in Fig. 2.

Results and discussion

The problem of the identity of 5-HTP decarboxylase with DOPA decarboxylase is first dealt with on the basis of the data from the inhibition experiments. As seen in Fig. 1 the decarboxylations of DOPA and 5-HTP were

inhibited one by another. An analysis according to LINEWEAVER-BURK (1934) revealed that the inhibition was competitive. This finding may be explained in two ways.

1) 5-HTP and DOPA interacted at the same active sites of one enzyme.

2) 5-HTP and DOPA attached to the active sites of two different enzymes, *i. e.* 5-HTP decarboxylase and DOPA decarboxylase, and thus interfered with each other's decarboxylation.

For the understanding of the reasoning the decarboxylation of DOPA alone and in the presence of 5-HTP will first be discussed.

If the enzyme preparation is allowed to act on DOPA we can set up the usual scheme of reaction.

$$DOPA + E \underset{k_{2D}}{\overset{k_{1D}}{\rightleftharpoons}} DOPA \cdot E \xrightarrow{k_{3D}} DA + CO_2 + E$$

and the equation for the reaction rate is

$$1/v_{DA} = \left(K_D/V_{DA}\right)\left(1/DOPA\right) + 1/V_{DA} \quad (1)$$

$$\text{where } K_D = (k_{2D} + k_{3D})/k_{1D}$$

v_{DA} stands for observed rate of dopamine formation; V_{DA} for the corresponding maximal rate; "DOPA" for the concentration of DOPA.

We can get K_D experimentally by varying the concentrations of DOPA and determination of the corresponding decarboxylation rates. Such experiments were performed and K_D was found to be 7.4×10^{-5}.

We now consider the experiments where mixtures of DOPA and 5-HTP were added. As earlier concluded 5-HTP appears to attach to the same sites of the DOPA decarboxylase as does DOPA and thus prevents DOPA to combine with some of these sites. For this inhibition we can write a new reaction scheme:

$$DOPA + E \underset{k_{2D}}{\overset{k_{1D}}{\rightleftharpoons}} DOPA \cdot E \xrightarrow{k_{3D}} E + DA + CO_2$$

$$HTP + E \underset{k_{2H}}{\overset{k_{1H}}{\rightleftharpoons}} HTP \cdot E \xrightarrow{k_{3H}} E + HT + CO_2,$$

and the rate of decarboxylation of DOPA can be obtained from the equation (for derivation see REINER 1959, p. 169)

$$1/v_{DA} = (1/V_{DA}) \times [(1 + HTP/K_{5\text{-}HTP})\, K_D \times (1/DOPA) + 1] \quad (2)$$

$$\text{where } K_{5\text{-}HTP} = (k_{2H} + k_{3H})/k_{1H};\ K_D = (k_{2D} + k_{3D})/k_{1D}$$

The formulas are written as if 5-HTP can be decarboxylated at this site. This is of no importance for the following as the formula (2) is valid whether k_{3H} is zero or not.

Using the values found in Fig. 1 $K_{5\text{-}HTP}$ can now be calculated. $K_{5\text{-}HTP}$ obtained in this way was found to be 1.3×10^{-4}.

We now turn to the decarboxylation of 5-HTP and follow the same mode

Table I. Enzyme-inhibitor dissociation constants of o-tyrosine, m-tyrosine and caffeic acid

Inhibitor	K_i*	
	DOPA as substrate	5-HTP as substrate
o-tyrosine	7×10^{-4}	7×10^{-4}
m-tyrosine	4×10^{-4}	4×10^{-4}
caffeic acid	3×10^{-4}	4×10^{-4}

* K_i was calculated according to eq. (2).

of reasoning. $K_{5\text{-}HTP}$ was found to be 1.8×10^{-4} at the direct determination according to formula (1). K_D as calculated as inhibition constant for the decarboxylation of 5-HTP was found to be 7.1×10^{-5}. It is thus seen that the values of $K_{5\text{-}HTP}$ and K_D calculated as inhibition constants as described above are the same as those obtained at the direct determinations. This finding is in agreement with the first mentioned assumption *i. e.* that the two amino acids are decarboxylated on the same sites of one enzyme. This thesis has also been tested in another way. m-tyrosine, o-tyrosine and caffeic acid not only do inhibit the decarboxylation of DOPA but also that of 5-HTP. The inhibitions are of the competitive type. If DOPA and 5-HTP are decarboxylated by two different enzymes both enzymes are inhibited by these substances. Even if it were so the degree of inhibitions of the two enzymes is not likely to be the same. Now using the formula (2) the inhibition constants for the two cases could be experimentally determined. The results are found in Table I. As will be seen the constants for the inhibitions of the DOPA and 5-HTP decarboxylation are roughly the same, which favours the assumption that we are dealing with only one enzyme.

The possibility that the DOPA and 5-HTP are competing for the common coenzyme, pyridoxal phosphate, seems to be excluded, since D-DOPA which reacts with pyridoxal phosphate as readily as L-DOPA but is not a substrate for DOPA decarboxylase does not inhibit the decarboxylation of 5-HTP.

Fig. 2 shows a fractionation of a rabbit kidney cortex preparation on a DEAE cellulose column. The preparation had been obtained from the extract by means of precipitations with ammonium sulphate. As will be seen the chromatographic distribution of DOPA and 5-HTP decarboxylase activities was very similar.[1] The ratio of the two activities throughout the peak was practically constant and equal to that found in crude extracts. Although the specific activity of the purest eluate was only about 40 times that of the homogenate the distribution strongly favours the assumption that we are

[1] This peak also contained all the histidine decarboxylase activity present in the extract. This fact together with the observation that caffeic acid was found to inhibit the decarboxylation of histidine has prompted a study on the relationships between DOPA decarboxylase and histidine decarboxylase.

dealing with one enzyme. The results are in good agreement with those of WERLE (1959) who found that a 20 fold purification of a similar enzyme preparation did not give any change in the relative activities.

There are thus three independent types of evidence that DOPA and 5-HTP decarboxylase are one enzyme: 1) They have the same distribution in tissues. 2) Their activity ratio has been found not to vary during fractionation by means of precipitations or chromatography. 3) The substrates inhibit each other's decarboxylation; the constants being the same as could be expected if they were competitive substrates.

Against these data stand the results of CLARK *et al.* (1954). Their findings do however not exclude the other possibility. The varying ratios of the two enzyme activities found by these authors may be explained by the marked instability of some of their preparations. It is possible that the activities were destroyed to different degrees when estimated at the two pH values used. The difference in coenzyme requirement may be due to the fact that DOPA combines with the coenzyme in a different way than 5-HTP. Nor is the difference in pH optima, as generally accepted, a reliable indication that the two substrates are attacked by two individual enzymes.

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Acta physiol. scand. 1960. 49. 370—375

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On the Role of Monoamine Oxidase for the Inactivation of Dopamine in Brain

By

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Received 15 February 1960

Abstract

ROSENGREN, E. *On the role of monoamine oxidase for the inactivation of dopamine in brain.* Acta physiol. scand. 1960. *49.* 370—375. — Evidence is presented that monoamine oxidase is involved in the metabolism of dopamine in brain. Thus 3,4-dihydroxyphenylacetic acid has been demonstrated to occur in the brains of three species investigated, including man. That this acid was derived from dopamine after oxidative deamination seemed probable as it was found to be localized in the same areas of the brain as dopamine and it disappeared after inhibition of monoamine oxidase with iproniazid. The capacity of this enzyme in brain seemed to be sufficient for the plausible inactivation rate of dopamine in this tissue.

The route of inactivation of catechol amines in tissues has long been a matter of debate. The discovery that monoamine oxidase, which is widely distributed in higher animals, also could metabolize catechol amines (BLASCHKO, RICHTER and SCHLOSSMANN 1937) at first led to the assumption that this enzyme was responsible for the inactivation of these physiologically active amines. Later on many serious objections against this thesis were aroused. The most important of them were that catechol amines were only slowly attacked by monoamine oxidase and that monoamine oxidase inhibitors did not significantly potentiate the effects of injected catechol amines. Further the effect of inhibition of monoamine oxidase on the brain catechol amine level was found to vary greatly. Thus administration of the monoamine oxidase inhibitor iproniazid (Marsilid) resulted in an increase in the brain noradrenaline content in rats (PLETSCHER 1957) while in rabbits there was no obvious effect (CARLSSON *et al.* 1957; BERTLER and ROSENGREN 1958). In cats

Table I. DOPAC in normal rabbit brain and after administration of drugs (μg/g).

	Brain stem	Corpus striatum	Cerebral hemispheres	Cerebellum
Control	0.01	0.48	0.05	0.05
Iproniazid (100 mg/kg) 16 h	—	0.07	—	0.01
Reserpine (1 mg/kg) 16 h	0.08	0.44	—	—
DOPA (100 mg/kg) 30 min.	0.61	1.64	0.43	0.30

even a decrease of the noradrenaline concentration in the hypothalamus has been observed (VOGT 1959). In this laboratory it was found, however, that pretreatment of rabbits with iproniazid prevented the effect of reserpine on noradrenaline in brain, suggesting that this enzyme had something to do with the catechol amine metabolism (CARLSSON *et al.* 1957). This was confirmed shortly afterwards by SHORE *et al.* (1957) and PLETSCHER (1957). Another observation pointing in the same direction was that the central actions of injected 3,4-dihydroxyphenylalanine (DOPA) was strongly potentiated if the rabbits were pretreated with iproniazid (CARLSSON *et al.* 1958). Recently another way for the inactivation of catechol compounds in tissues was proposed since it was found that after administration of such substances to animals a considerable part was excreted as 3-O-methylated derivatives (SHAW, MCMILLAN and ARMSTRONG 1957; AXELROD *et al.* 1958). These and other data suggested that O-methylation might be the first step in the catabolism of catechol amines in brain as well as other tissues (AXELROD, ALBERS and CLEMENTE 1959).

In the present investigation a somewhat different approach to elucidate the role of monoamine oxidase for the catabolism of brain dopamine has been undertaken. If dopamine is oxidatively deaminated, the deamination product should be 3,4-dihydroxyphenylacetaldehyde, which is readily oxidized to the corresponding acid, 3,4-dihydroxyphenylacetic acid (DOPAC). The occurrence and distribution of DOPAC in tissues may therefore give valuable information on the role of monoamine oxidase in the dopamine metabolism. Although this acid has been found to be present in body fluids and some tissues, including brain (EULER 1958), the available data concerning the presence of DOPAC in brain are very sparse.

Experimental

Pieces of the brains of different species were homogenized in twice their weights of 10 per cent metaphosphoric acid. The homogenates were centrifuged for 15 min at 24,000 × *g*, and the supernatants were filtered. The residues were reextracted twice with the original volumes of metaphosphoric acid. The combined filtrates were shaken with the same volumes of ethyl acetate after saturation with sodium chloride. The ethyl acetate was sucked off and evaporated to dryness *in vacuo*. The dry residue was

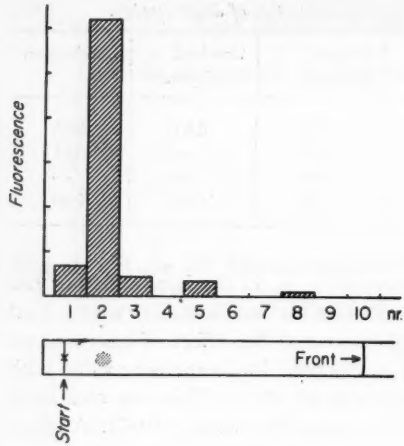

Fig. 1. Quantitative paper chromatography of an extract of the corpus striatum of the pig. Fluorescent wave-length 565 mμ. Activating wave length 435 mμ. For explanation see text.

extracted with a few drops of ethyl acetate and chromatographed on paper (Whatman no. 1) together with a reference containing DOPAC. The solvent system generally used was benzene-propionic acid-water (2 : 2 : 1) (ARMSTRONG, SHAW and WALL 1956). In some cases also other systems were used. After development, the paper was divided into two parts, one containing the reference, the other the brain extract. The reference spot was visualized by spraying with a potassium ferricyanide solution (JAMES 1948). The other part was cut into small strips and eluted with 5 ml 0.01 N hydrochloric acid. Two ml of a mixture of redistilled ethylene diamine and 4 M ammonium chloride (1 : 1.3) according to KÄGI, BURGER and GIGER (1957) was added to each of the eluates in a glass-stoppered test tube which was then placed into a water bath (63° C) for 20 min. Eight ml n-butanol and 2 g sodium chloride was added and the test tubes shaken. Under these conditions DOPAC and ethylene diamine formed a highly fluorescent condensation product which was retained in the water-phase. The fluorescence intensity was measured in an Aminco-Bowman spectrophotofluorometer at 435 mμ activating wave-length and 565 mμ fluorescence wave-length (uncorrected values).

The normal distribution of DOPAC in rabbit brain is found in Table I, in which the concentrations of this acid after the administration of D,L-3,4-dihydroxyphenylalanine, reserpine and iproniazid are also given.

In another set of experiments the capacity of brain tissue to form DOPAC from dopamine was investigated. In these experiments whole brains of rabbit or pieces of human brain were homogenized in ice cooled 1/15 M phosphate buffer, 2.5 ml per g. Half a ml of the homogenate was incubated with varying concentrations of dopamine at 37° C for 30 min. The incubation was carried out in an atmosphere of ordinary air and pH 6.5. This pH value was slightly below optimum but was preferred as otherwise the autoxidation of dopamine was considerable. At the end of incubation DOPAC was determined as described above. For the present purpose this method for determination of monoamine oxidase activity is probably more reliable. In preliminary experiments it was found that DOPAC was not further metabolized in the brain tissue. In Fig. 3 the rates of DOPAC formation has been plotted against the concentration of dopamine.

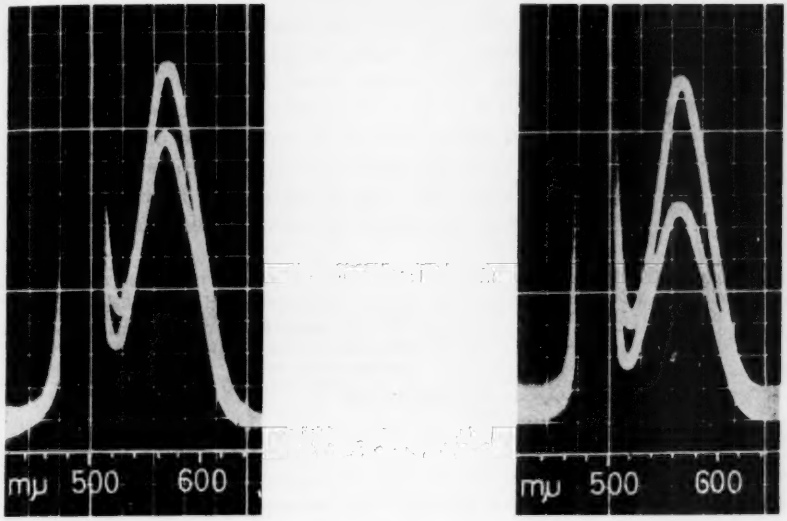

Fig. 2. Fluorescence spectra of DOPAC (upper curves) and of material from the corpus striatum of man (left) and rabbit (right) after condensation with ethylene diamine. Activating wave length 435 mμ.
Note. The rabbits had been given 1 mg reserpine 16 h. before death.

Results and discussion

The method used for the determination of DOPAC in tissues has been tested in several distribution and recovery experiments. The yield of the three successive extractions of the brain tissue with metaphosphoric acid as described above was 85 per cent, about 50 per cent of the DOPAC present in the tissue being removed in each extraction. It was found that about 90 per cent of DOPAC could be extracted from 10 ml of the metaphosphoric acid solution saturated with sodium chloride by shaking with the same volume of ethyl acetate for 4 min. No appreciable loss in the other steps of the procedure was found to occur.

Using this technique DOPAC was found to be present in the corpus striatum of the rabbit (Table I, Fig. 2) where the concentration was about 0.5 μg per g. DOPAC in a concentration of about 0.3 μg per g was also detected in this brain region of pig and in man (Fig. 1 and 2) two hours after death. In other parts of the brain no significant amounts could be detected. Thus DOPAC was found to be localized in the same brain areas as dopamine (Bertler and Rosengren 1959). Injection of D,L-3,4-dihydroxyphenylalanine into rabbits caused a considerable increase of DOPAC in the brain. The highest concentrations were observed in the corpus striatum and the brain stem. The last

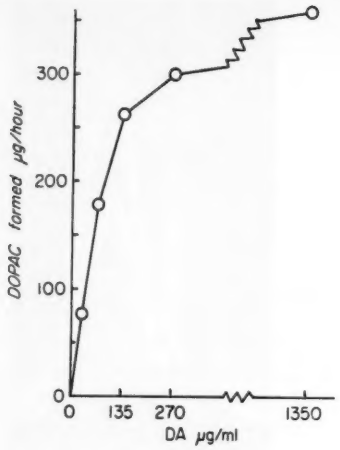

Fig. 3. Variation of rate of DOPAC formation with the concentration of dopamine. Each vessel contained rabbit brain homogenate (pH 6.5) corresponding to 0.17 g tissue. Temperature 37° C. Final volume 3 ml.

mentioned fact indicates that DOPAC was not carried to the brain by the blood but was formed in the brain itself, the distribution of DOPAC being the same as that of excess dopamine after administration of D,L-3,4-dihydroxyphenylalanine (BERTLER and ROSENGREN 1959).

The alternative way of DOPAC formation in the body proposed by SHAW *et al.* (1957) *i. e.* transamination of 3,4-dihydroxyphenylalanine followed by decarboxylation did not seem to be responsible for the DOPAC found in brain. After pretreatment of the animals with iproniazid, 3,4-dihydroxyphenylalanine did not give any increase of the concentration of DOPAC. Administration of iproniazid only, caused DOPAC to disappear almost completely from the brain. Since iproniazid, as far as is known, does not inhibit these last mentioned enzymic reactions the effect of iproniazid seemed to be due to inhibition of monoamine oxidase.

Reserpine which is known to deplete the body stores of catechol amines (CARLSSON *et al.* 1957, 1958) apparently did not influence the concentration of DOPAC in brain. It might be argued that a product of reserpine or its solvent might have similar properties as DOPAC and thus invalidate the analysis. However, the substance observed after reserpine was found to have the same R_f value and the same fluorescence characteristics as DOPAC (Fig. 2) and therefore probably was DOPAC. This was in agreement with the view that reserpine does not influence the formation of dopamine in tissues but prevents the normal storage of this amine.

From Fig. 3 it will be seen that even at low concentrations of dopamine the formation of DOPAC was sufficiently rapid to account for the probable inactivation rate of dopamine in brain (CARLSSON, LINDQVIST and MAGNUSSON 1959).

In summary, it can be said that DOPAC, which is the product expected to be formed from dopamine by the action of monoamine oxidase has been found to occur in brains of the species investigated (rabbit, pig, and man). The capacity of monoamine oxidase in brain tissue to destroy this amine as studied *in vitro* seems to be sufficient to account for the inactivation of these amines *in vivo*. It therefore seems probable that dopamine is, at least partly, metabolized by means of monoamine oxidase. This does not exclude the possibility that dopamine under normal conditions also is methylated.

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Effect of Reserpine on the Adrenal Medulla of Sheep

By

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Abstract

HILLARP, N.-Å. *Effect of reserpine on the adrenal medulla of sheep.* Acta physiol. scand. 1960. *49.* 376—382. — The changes found in the sheep adrenal medulla on reserpine treatment were, though more pronounced, very similar to those found on indirect stimulation with insulin induced hypoglycemia. On both treatments the mechanism holding the equivalent amounts of amines and adenosinephosphates within the medullary granules is affected in such a way that the amines are released and the adenosinephosphates are broken down and/or released at the same time. In neither case do amines or adenosinephosphates accumulate in the extragranular cytoplasm and the adenine moiety of these phosphates is rapidly disposed of.

The body stores of 5-hydroxytryptamine are depleted by reserpine, probably by interfering with the storage mechanism (see SHORE *et al.* 1957) and causes the cathecholamines stored in the adrenal medulla, adrenergic nerves and brain to disappear (BERTLER, CARLSSON and ROSENGREN 1956; CARLSSON and HILLARP 1956; HOLZBAUER and VOGT 1956).

Since the mode of action of reserpine on the adrenal medulla is not properly understood (see Dissussion), some of the changes it produces in the medulla were studied. The changes of the adenosinephosphates and their intracellular distribution received particular attention, since there is good evidence that the catecholamines are stored together with these phosphates in the amine granules.

Methods

Male sheep (body wt. 25 to 26 kg) were used. Two experiments were performed, and in each two animals were used. One animal was given reserpine (Serpasil, Ciba, 2 or 4 mg/kg of body wt. subcutaneously), and the other served as a control. Between 13 and 14 hours after the injection, the animals were killed and the adrenals treated in the way described in a previous paper (CARLSSON, HILLARP and HÖKFELT 1957).

Cell fractionation. The weighed adrenal medulla was homogenized in 8 ml of 0.3 M sucrose (*cf.* HILLARP, LAGERSTEDT and NILSON 1953). The homogenate was freed from cells, nuclei, connective tissues etc., by centrifugation at 800 × g for 6 min. The sediment was re-homogenized in 8 ml of sucrose and re-centrifuged once (Fraction S). A large granule fraction (G) was obtained from the combined supernatants by centrifugation at 23,000 × g for 20 min. The supernatant (Fraction A) was carefully sucked off. The fractions were extracted with perchloric acid and analyzed for catecholamines, proteins, inorganic and acid-labile phosphorous (see CARLSSON, HILLARP and HÖKFELT 1957).

Determinations of nucleotides and related compounds. Aliquots of the G extracts were analyzed by means of ion exchange chromatography and high voltage paper electrophoresis (see HILLARP and THIEME 1959). It must be noted, however, that the methods used yield values for the adenosinephosphates which are too high owing to the presence of smaller amounts of other nucleotides (HILLARP 1960).

The A extracts were only passed through a small cation exchange column (Dowex 50, pH 6). The ultraviolet spectrum of the effluent was taken at pH 2. It showed a pronounced maximum at 257 to 260 mμ and minimum at 230 to 235 mμ and may thus possibly give a rough estimate of the amounts of adenine compounds present. At any rate the maximal amounts could be calculated. The substances retained on the column were eluted with N HCl. Calculations based on the spectrum and catecholamine content of the eluates showed that at most small amounts of nucleosides or their bases could have been present.

Results

Catecholamines

The reserpine treatment caused a marked decrease in the catecholamine content of the adrenal medulla (Table I). Even if the medullary tissue did increase in weight, the drop in the amines must have been at least 90 per cent.

In the two normal animals 89 to 90 per cent of the catecholamines were recovered in the granule fraction. About the same intracellular distribution of the amines was found in one of the animals treated with reserpine. In the other animal a fair proportion (31 per cent) of the amines was recovered in the extragranular cytoplasm, but this shift in the distribution might have occurred *post mortem.*

Adenosinephosphates, inorganic and acid-labile phosphates

The large granule fraction (containing *i. a.* the amine granules and mitochondria) in the two normal sheep showed about the same content and pattern of adenosinephosphates (Table I) as did pure amine granules isolated from the adrenal medulla of two other ruminants (cow and goat; HILLARP and THIEME 1959).

Table I. Content and intracellular distribution of catechol amines, adenosinephosphates, acid-labile and inorganic phosphates in adrenal medulla of normal (N) and reserpine treated (R) sheep

Figures within parentheses are μmoles/μmole amines.

Fraction S: Sediment after centrifugation at 800 × g for 6 min. (connective tissue, whole cells, cell nuclei etc.).

Fraction G: Sediment after centrifugation at 23,000 × g for 20 min. (amine granules, mitochondria, some microsomal material).

Fraction A: Supernatant fluid after last centrifugation ("soluble" cytoplasm, microsomal material).

	Medulla		Fraction G							Fraction S
	Weight mg	Amines mg/g	Amines μmoles	Amines Per cent of (G+A)	ATP μmoles	ADP μmoles	AMP μmoles	P_8 μmoles	P_0 μmoles	Amines μmoles
N1	417	10.2	19.9	90	4.70 (0.235)	0.585 (0.029)	0.275 (0.014)	10.5	2.30	1.51
N2	184	10.3	9.16	89	2.15 (0.235)	0.320 (0.035)	0.105 (0.011)	4.90	1.30	0.30
R1	524	0.45	0.97	84	0.215 (0.220)	<0.09	<0.05	0.7	<0.3	0.16
R2	273	0.55	0.50	69	0.08 (0.16)	<0.07	<0.04	0.3	<0.3	0.11

		Fraction A					Degree of Homogenization Per cent
				Adenine compounds μmoles			
N1		2.21	10	< 2.1	0	6.20	94
N2		1.14	11	< 1.7	0	2.60	97
R1		0.19	16	< 0.12	0	4.85	88
R2		0.22	31	< 0.15	0	2.85	87

The reserpine treatment was followed by a disappearance of ATP in the granule fraction, which was about equal to that of catechol amines. There was a considerable decrease in ADP and AMP, too, but only maximal figures for the content of these phosphates could be calculated, the amounts being too small for accurate determination. The drop in ATP and ADP was accompanied by the disappearance of the acid-labile phosphates in the granules. No split products from the adenosinephosphates (inorganic phosphate, adenosine, adenine) were demonstrable in the granules.

Fraction A ("soluble" cytoplasm and microsomal material) in the normal animals contained large amounts of ultraviolet absorbing substances with a spectrum suggesting them to consist mainly of adenine compounds (see Methods). From these measurements the maximal content of adenine compounds was

Table II. Content and intracellular distribution of proteins in adrenal medulla of normal (N) and reserpine treated (R) sheep

Cell fractions: see text to Table I.

Cell fraction	Protein N: mg Observed				Distribution of Protein N calculated for complete homogenization Per cent			
	N1	N2	R1	R2	N1	N2	R1	R2
G	1.70	0.74	1.00	0.64	28	27	16	20
A	2.75	1.20	3.45	1.85	45	44	54	55
S	2.05	0.87	2.70	1.30	27	28	30	25
Total: mg per g medulla	15.6	15.2	13.5	13.9				

calculated in order to reveal any accumulation of split products from the ATP disappearing from the granules on reserpine treatment. It is evident (Table I), however, that no accumulation took place. Moreover, the bulk of the substances with high absorbancy at 250 to 270 mμ disappeared.

The possible splitting of ATP on amine release might result in an accumulation of inorganic phosphate. However, this phosphate decreased in the granules, and no evident increase occurred in fraction A (Table I).

Proteins

The data for the protein content of the various fractions are found in Table II. Since the degree of homogenization of the medullary tissue differed from animal to animal (Table I) figures calculated for a complete homogenization are also given.

The values found for the protein distribution appear to indicate that reserpine treatment caused a decrease in the proteins in the granules and increase in the "soluble" cytoplasm. The loss of proteins from the granule fraction may be only apparent, however, since an increase in weight of the medulla and in cytoplasmic proteins may have occurred (*cf.* CARLSSON, HILLARP and HÖKFELT 1957).

Discussion

Apparently contradictory results have been obtained in studies on the problem whether reserpine acts directly on the adrenal medullary cells or indirectly via their secretory nerves. In the rabbit the action seems to be predominantly central (KRONEBERG and SCHÜMANN 1957). In the cat (CARLSSON 1958) and rat (KRONEBERG and SCHÜMANN 1957, CALLINGHAM and MANN 1958 b, MIRKIN 1958), however, neither cutting of the splanchnic nerves nor transection of

the spinal cord at C_6 prevent the amine depletion caused by reserpine. No reasonable hypothesis accounting for all observations made (*cf.* KRONEBERG and SCHÜMANN 1957) has as yet been put forward to explain the lack of agreement between the results. Undoubtedly, however, reserpine has both a central and a peripheral action, though possibly of varying degrees, in the different animal species.

It seems most reasonable — though no conclusive evidence is available (*cf.* CARLSSON *et al.* 1957, KRONEBERG and SCHÜMANN 1958) — that the amine depletion produced by the direct action of reserpine on the adrenal medulla is a result of a release and secretion of the amines. This and the observation that reserpine does not apparently prevent a normal amine resynthesis (CALLINGHAM and MANN 1958 a and b, MIRKIN 1958) suggest that its peripheral action is on the storage mechanism. It has previously been assumed that reserpine acts primarily by destroying the storage mechanism of 5-hydroxytryptamine in blood platelets (SHORE *et al.* 1957, HUGHES, SHORE and BRODIE 1958).

The considerations set forth above favour the view that a closer analysis of the mode of action of reserpine on the adrenal medulla might give valuable clues concerning the amine storage mechanism. The experiments in the present paper were, however, only intended to give some preliminary information on possible changes in the stored amines and adenosinephosphates as compared with the changes found on indirect stimulation produced by insulin hypoglycemia (CARLSSON, HILLARP and HÖKFELT 1957).

In spite of the fact that the reserpine treatment depleted the medullary granules of their stored catechol amines to more than 90 per cent during a relatively short period of time, the intracellular distribution of the amines in one of the animals clearly showed that this high depletion rate need not result in an accumulation of amines in the extragranular cytoplasm. Since the medullary cells have not been found to contain any highly active systems catabolizing catechol amines — the low amine oxidase activity in the medulla probably belongs to ganglion cells (FRANCIS 1954) — and since no accumulation could be demonstrated of ultraviolet absorbing substances indicating an intracellular degradation (by *e. g.* the cytochrome oxidase system) of the amines that had disappeared, it seems reasonable to assume that the amines bound to the granules were liberated and then diffused or were transported out of the cells.

It has previously been shown that an amine liberation from the granules produced by stimulation of the adrenal medulla via the secretory nerves is accompanied by an approximately equal drop in the stored ATP (CARLSSON and HILLARP 1956, CARLSSON, HILLARP and HÖKFELT 1957). SCHÜMANN (1958 b) and KIRPEKAR, GOODLAD and LEWIS (1958) showed that reserpine also has this effect. The same result was obtained in the present study, which also demonstrated that the bulk of ADP and AMP present in the granules also disappeared along with the amines. A total amount of about 10 μmoles of adenosinephosphates per g medulla was lost from the granules without leaving any detect-

able degradation products (*e. g.* adenine, adenosine, hypoxanthine, inosine, inorganic or acid-labile phosphates). The adenosinephosphates must either have been rapidly broken down and/or secreted out of the cells since the adenine moiety completely disappeared, and an incorporation of such high amounts into acid-insoluble adenine compounds does not seem likely. There is evidence that the adrenal medulla contains an adenylic acid deaminase (HILLARP 1958), but no accumulation of inosinic acid, inosine or hypoxanthine could be detected in the medulla of the reserpine treated animals (*cf.* HILLARP 1960). There was, however, not only no accumulation of adenine compounds or closely related substances in the extragranular cytoplasm, but on the contrary a very pronounced decrease in substances showing an ultraviolet absorption suggesting the presence of such compounds in this part of the normal cell.

The changes found in the adrenal medulla on reserpine treatment are, though more pronounced, very similar to those found on indirect stimulation with insulin induced hypoglycemia. On both treatments the mechanism holding the equivalent amounts of amines and adenosinephosphates kept within the granules is affected in such a way that the amines are released and the adenosinephosphates are broken down and/or released at the same time. Furthermore, in neither case do amines or adenosinephosphates accumulate in the extragranular cytoplasm and the adenine moiety of these phosphates is rapidly disposed of. Since it is unknown, however, whether reserpine acts predominantly directly of indirectly on the adrenal medulla of sheep, further experiments on denervated glands are needed.

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